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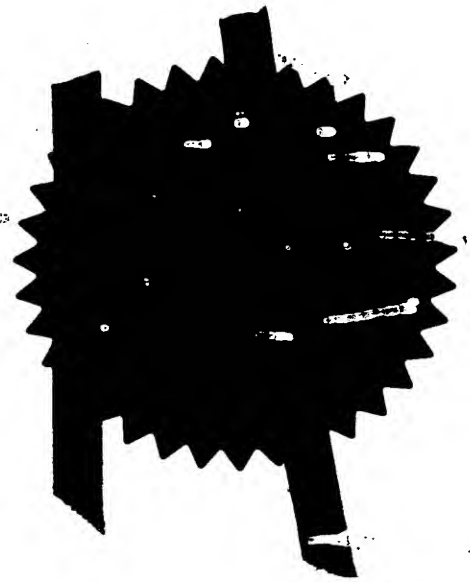

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Dated 18th July 2000

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2.	Patent application number *(The Patent office will fill in this part)*	**9929547.9**
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	**GLAXO GROUP LIMITED** **GLAXO WELLCOME HOUSE** **BERKELEY AVENUE** **GREENFORD** **MIDDLESEX** **UB6 ONN** **GB**
	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its corporation	**GB**
4	Title of the invention	**DNA CONSTRUCTS**
5	Name of your agent *(if you know one)*	**MARION L REES (SEE CONTINUATION SHEET)**
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	**GLAXO WELLCOME PLC** **GLAXO WELLCOME HOUSE, BERKELEY AVENUE** **GREENFORD, MIDDLESEX** **UB6 ONN, GB**
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DNA Constructs

The present invention concerns novel DNA constructs, host cells comprising (harbouring) said constructs, methods and processes for the production of a desired protein, particularly a therapeutically useful protein, DNA vaccines and other medicaments comprising said constructs and methods of gene therapy using said constructs.

Eukaryotic Initiation Factor 4A (eIF-4A) is a 43kD protein with RNA helicase activity that mediates binding of mRNA to the 40S subunit of the ribosome. In the mouse genome there are two functional eIF4A genes, eIF4AI and eIF4AII (1,2). The two eIF4A genes are differentially regulated in the mouse with $eIF4A_I$ mRNA being expressed at higher levels than the $eIF4A_{II}$ mRNA in dividing cells with the $eIF4A_{II}$ mRNA being preferentially expressed in non dividing cells (3). In the human genome the homologous eIF-4AI and eIF-4AII genes have been mapped to chromosomes 17p13 and 18p11.2 respectively (4,5).

In common with many other 'housekeeping genes' the gene encoding eIF-4AI must be expressed in every mammalian cell type at approximately similar levels. Many housekeeping gene promoters do not contain TATA box sequences to direct transcription initiation but rather have GC rich promoters that contain binding sites for the transcription factor Sp1 and initiator elements (6). Many housekeeping gene promoters contain a higher than expected frequency of the dinucleotide CpG and these regions are not as extensively methylated as other CpG dinucleotides elsewhere in the genome (7). Such Methylation Free Islands (MFI) are often associated with transcriptional regulatory regions of genes and are thought to act by maintaining adjacent chromatin regions in an open configuration (8).

Cloning of the genes defective in human monogenic diseases such as the Cystic Fibrosis has raised the possibility of introducing a non mutated form of the Cystic Fibrosis Transmembrane Regulator (CFTR) gene into the appropriate cell types of affected individuals to restore normal function. In order for such somatic gene therapy protocols to be useful the therapeutic gene of interest must be stably expressed at a level similar to or higher than that of the mutated gene. Nearly

all somatic gene therapy vectors used to date have relied upon transriptional regulatory regions derived from viruses such as the SV40 enhancer, retroviral Long Terminal Repeat sequences or the promoter/enhancer element of the human Cytomegalovirus immediate early gene. While recombinant retroviruses can efficiently infect dividing primary cells gene expression from integrated proviral genomes is often unstable (9). Similar problems have been encountered with long term gene expression using episomal viral vectors (10).

More recently *in vivo* delivery of plasmid DNA containing antigen genes under the control of viral promoters has been used to elicit humoral and cell mediated immune responses in experimental animal models (11). It is not yet clear what properties the ideal mammalian expression vector for DNA vaccination should have in order to elicit an effective immune response. Many currently used viral promoters do not direct high levels of gene expression in professional antigen presenting cells. Thus a promoter which can drive expression in macrophages and other cell lines where CMV gives poor expression, e.g. prostate cancer cells, would be desirable for both genetic vaccination and gene therapy.

It is an object of the present invention to provide alternatives to the currently used viral promoters.

In accordance with the present invention there is provided a (novel) DNA construct comprising a transcriptional regulatory sequence operatively linked to a (heterologous) gene of interest which sequence comprises a polynucleotide derivable from the (wild type) eIF4AI gene promoter or fragment thereof.

In accordance with a further aspect of the present invention there is provided a novel DNA construct comprising a transcriptional regulatory sequence operatively linked to a gene of interest which regulatory sequence comprises a polynucleotide derivable from the eIF4AI gene promoter or fragment thereof and at least one intron sequence of the eIF4AI gene.

In accordance with a further aspect of the present invention there is provided a novel DNA construct comprising a transcriptional regulatory sequence operatively linked to a heterologous gene of interest which regulatory sequence

comprises a polynucleotide derivable from the eIF4AI gene promoter or fragment thereof and a polynucleotide derivable from at least one intron sequence of the eIF4AI gene wherein the intron sequence is selected from the group consisting of; intron 1, 2, 3, 5, 6, 7, 9.

In accordance with a further aspect, there is provided a host cell comprising (harbouring) a DNA construct as hereinbefore described.

In accordance with a further aspect, there is provided a process for the production of a protein of interest, which process comprises the step of culturing said cells as hereinbefore described.

In accordance with another aspect, there is provided a DNA vaccine comprising the construct as hereinbefore described. Methods for the treatment, including prophylatically where appropriate, of diseases or disorders comprising the step of administrating said vaccine are also provided. Gene therapy methods comprising the step of administrating said construct are also provided.

The present invention provides DNA constructs that are capable of expressing a gene of interest over a longer time period than provided for by the viral promoters such as the human CMV promoter. Such expression characteristics are particularly beneficial where long term expression is required such as in gene therapy, vaccination treatments and in the commercial production of proteins.

In preferred embodiments, the construct of the present invention is non-chromosomal e.g. a phage, plasmid, virus, minichromosome or transposon. Of these, plasmid is particularly preferred. The transcriptional regulatory sequence comprises a polynucleotide derivable from eIF4AI promoter or fragment thereof. SEQ.I.D.NO:38 sets forth the sequence of the human eIF4A1 promoter from position -526. By the term "derivable" it is intended to convey a source not only in the sense of it being the physical source for the material but also to define material which has structural and/or functional characteristics which correspond to those materials but which does not originate from the reference source. The polynucleotide is preferably derived from a mammalian source,

particularly murine, rattus or human, preferably human. Fragments of the eIF4AI promoter may be used in place of, or in addition to the full length promoter sequence so long as they retain the biological characteristic of a promoter when incorporated into constructs of the present invention. Indeed, the present inventors have demonstrated that such fragments may be obtained from the full length eIF4AI promoter sequence. Accordingly, such fragments include -526EIF, -371EIF, -271EIF, -193EIF, -120EIF, -98EIF, -69EIF and -40EIF. Variants of the wild type eIF4AI promoter sequence are also envisaged. Such variants maybe naturally occurring variants which may have a substitution, deletion or insertion of one or more bases. Variants also include non-naturally occurring variants in which one or more bases have been added, substituted, inserted, deleted, rearranged or modified yet retain promoter characteristics. Also encompassed within the scope of the present invention are variants having at least 90% identity with the eIF4AI promoter or fragment thereof, e.g. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% identity.

In preferred forms, the transcriptional regulatory sequence comprises a polynucleotide derivable from the eIF4AI promoter or fragment thereof and at least one intron (or fragment thereof) derivable from the eIF4AI gene. In particular, it is preferred that the intron is derivable from intron 1,2,3,5,6,7 or 9. Of these it is particularly preferred that the polynucleotide is derived from intron 1 of the eIF4AI gene. It is preferred that the intron polynucleotide is derived from a mammalian source, e.g. murine, rattus or human, preferably human. Variants of the intron sequence in which one or more bases have been added, substituted, inserted, deleted, rearranged or modified yet retain enhancer characteristics are also envisaged. Variants of the transcriptional regulatory sequence having at least 90% identity, e.g. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% are also provided. The present inventors have demonstrated advantage in utilising more than one intron derivable from eIF4AI gene. Thus, constructs may comprise a transcriptional regulatory sequence comprising a polynucleotide derivable from the eIF4A promoter or fragment thereof and a plurality of eIF4AI gene introns. The additional intron sequence may be the same or different from the preceding intron. It is preferred that the polynucleotide comprises the eIF4AI promoter sequence or fragment thereof and eIF4AI intron sequence or fragment thereof arranged in tandem in forward

or reverse orientation. The intron sequence and promoter sequence may be co-terminus or spaced apart. The intron sequence may be upstream or more preferably downstream (in terms of reading frame) of the eIF4A promoter sequence. Chimaeric constructs e.g. one in which the polynucleotide comprises the eIF4A promoter or fragment thereof derived from a first species and the intron sequence derived from a second species are also envisaged. Preferably, the promoter sequence is produced by recombinant means, such techniques being standard and well known to those skilled in the art.

Constructs of the present invention may further comprise other elements such as a terminator sequence and a selectable marker, bacterial origin of replication, antibiotic resistance gene and a signal peptide gene for secretion if necessary. Constructs of the present invention are preferably incorporated into a host cell for gene expression although cell-free translation systems are not excluded. The incorporation of the constructs may be achieved by methods well known to those skilled in the art, e.g. P1 transduction. Suitable host cells maybe prokaryotic or eukaryotic, preferably eukaryotic, even more preferably mammalian. Host cells may be fully differentiated, pluripotent stem cells or other precursor cells. The present invention is particularly advantageous in directing high level persistent expression of a gene of interest in professional antigen presenting cells such as macrophages and dendritic cells and other cells that have been shown to be refractory to high level, persistent gene expression by viral promoters e.g. prostate and colorectal cancer cells.

Host cells transformed by constructs of the present invention may be used in the commercial production of proteins. Generally, a fermentation method is employed which involves submerging the cells in a culture medium contained within a suitable vessel. Following culturing at appropriate conditions, proteins secreted by host cells maybe recovered from the culture medium by standard techniques known to those skilled in the art. It is preferred that the protein is recovered to homogeneity. In addition transformed host cells of the present invention maybe utilised in therapeutic strategies for replacing or compensating for cell loss. For example, mammalian host cells for transplantation (which maybe autologous or allogenic or less preferred xenogeneic) maybe transformed *ex vivo* with a gene construct of the present invention in which the

gene of interest encodes dopamine for the treatment of Parkinson's disease although other cell loss diseases or disorders which may benefit from the present invention will be readily apparent to those skilled in the art. Viruses, particularly retroviruses and adenoviruses, incorporating the transcriptional regulatory sequence of the present invention are also envisaged. Embodiments in which the gene of interest encodes a prodrug converting enzyme are also provided.

Of particular interest is the use of the constructs of the present invention in DNA vaccines and gene therapy. Examples of DNA vaccines that may benefit from the present invention include tumour vaccines, hepatitis B and C, HIV, tuberculosis, HPV and HSV vaccines and vaccines directed at modifying chronic inflammatory reactions, such as MS, asthma, RA and Alzheimers, or directed at other biological pathways, such as vaccines for contraception or drug addiction.

For gene therapy treatments, constructs may be incorporated into a carrier such as liposomes. Typically such liposomes are cationic for example imidazolium derivatives (WO95/14380), guanidine derivatives (WO95/14381), phosphatidyl choline derivatives (WO95/35301), piperazine derivatives (WO95/14651) and biguanide derivatives. The construct may comprise a gene of interest such as CTFR or erythropoetin gene operatively linked to the promoter sequence. Thus a method of correcting or compensating for a disease or disorder whose etiology is characterised by a genetic aberration (such as cystic fibrosis) is provided, which method comprises the step of administrating to a mammalian patient in clinical need thereof a therapeutically effective amount of the construct, preferably incorporated into a carrier. In a particularly advantageous use, constructs of the present invention may be used in driving high level persistent gene expression in avelolar macrophages.

The promoter sequence of the present invention is operatively linked to a gene of interest. Genes of interest include genes encoding for therapeutic or non-therapeutic proteins. Therapeutic proteins include those whose large scale commercial production is hampered by the prohibitive cost of current techniques. The gene of interest may encode a protein which is heterologous with respect to the host cell, i.e. not normally expressed in the host cell. By way of illustration,

genes encoding for proteins such as interferon-β, factor VIII, erythropoetin, growth factors and cytokines or therapeutically effective fragments thereof, are all examples of genes of interest. Medicaments, particularly pharmaceutical compositions, comprising therapeutic proteins produced according to the present invention are also provided. The elf4A promoter is active in all cell types, i.e. it is a ubiquitously active promoter, and gives sustained high level expression in all cell types.

In accordance with further aspects of the present invention, isolated forms of introns 1 (SEQ.I.D.NO:31),2 (SEQ.I.D.NO:32),3 (SEQ.I.D.NO:33),5 (SEQ.I.D.NO:34),6 (SEQ.I.D.NO:35),7 (SEQ.I.D.NO: 36) and 9 (SEQ.I.D.NO:37) are provided. By the term "isolated" we mean that the nucleic acid as described herein exists in a physical milieu distinct from that in which it occurs in nature. For example, the nucleic acid may be isolated with respect to one or more materials it is associated with in the natural state. Thus in accordance with the present invention, there is provided an isolated polynucleotide comprising (or consisting essentially of) the sequence as set forth in SEQ.I.D.NO:31 or 32 or 33 or 34 or 35 or 36 or 37 or variant thereof. A recombinant DNA construct comprising a transcriptional regulatory sequence which regulatory sequence comprises a polynucleotide derivable from the isolated polynucleotide as described herein is also provided. Isolated polynucleotides as identified herein maybe used as enhancer elements in transcriptional regulatory sequences found in recombinant DNA constructs. In other aspects, a recombinant DNA construct comprising an transcriptional regulatory sequence operatively linked to a heterologous gene of interest which regulatory sequence comprises (or consists essentially of) a polynucleotide having a sequence as set forth in SEQ.I.D.NO:38 or variant thereof preferably further comprising a sequence as set forth in any one of SEQ.I.D.NO:31, 32,33,34,35,36,37 or variant thereof is provided.

In other aspects there is provided an isolated polynucleotide having a sequence as set forth in fig.9 at positions -2102 to -1082 or variant thereof.
There is also provided an isolated polynucleotide having a sequence as set forth in fig.9 at positions -1107 and -505 or variant thereof.

Suitable techniques for introducing the naked polynucleotide or vector into a patient include topical application with an appropriate vehicle. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology or Particle Mediated DNA Delivery and is described in US Patent No. 5371015). Here inert particles (such as gold beads) are coated with a nucleic acid, and are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin), for example by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

The constructs of the invention may be administered as pharmaceutical compositions, which are typically administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 1 µg/kg body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about 1 µg/kg to about 1 mg/kg body weight, daily. It will be appreciated that

optimum dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like.

In therapy or as a prophylactic, the construct may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 1 µg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

The present invention will now be illustrated by way of example only. It should be understood that these represent preferred embodiments of the invention. Various modifications and changes within the spirit and scope of the invention will be apparent to those skilled in the art.

Brief description of drawings.

Fig.1: illustrates the organisation of the human eIF4A1 gene. Coding sequence exons of the eIF4A1 and CD68 genes are represented by black boxes, introns by white boxes and 3' untranslated regions by shaded boxes. The position of Alu I repeat sequences is shown by horizontal arrows below the line. Vertical lines with arrowheads denote the transcription start sites of the two genes, poly A addition sites are represented by the symbol pA^+. Hatched boxes delineate the regions of the human eIF4A1 gene sequence which share homology with the murine eIF4A1 promoter and the position of the CpG rich methylation free island within intron 1. White and black boxes represent the positions of promoter fragments, (PCR A and PCR B respectively), assayed for transcriptional activity in fig.4.

Fig.2: DNA sequence of the promoter and exon 1 of the human eIF4A1 gene. DNA sequences are numbered with the putative transcription site denoted as +1. Potential transcription factor binding sites are underlined. The amino acids encoded by exon 1 are shown. eIF4A1 gene sequence was determined by DNA sequencing of the cosmid clone cosCD68C1 (Jones, E., Quinn, C.M., See, C.G., Montgomery, D.S., Ford, M.J., Kolble, K., Gordon, S., and Greaves, D.R. (1998), Genomics 53: 248-250).

Fig.3: Mapping the transcriptional regulatory sequences of the eIF4A1 promoter. The indicated luciferase reporter plasmids were introduced into CHO, 293 and RAW264.7 cells along with a beta-galactosidase reporter gene plasmid (pcDNA Beta gal) as described below. Cell lysates were prepared 16 hours after transfection and assayed for luciferase and beta galactosidase enzyme activities. Cell lysates were diluted in cell lysis buffer to ensure that all luciferase assays were within the linear range of the enzyme assay. The error bars represent the standard error of the mean of two independent transfection experiments.

Fig.4: Transcriptional regulatory sequences in eIF4A1 5' flanking sequence. Plasmid DNAs of the indicated plasmids (20μg) were introduced into RAW264.7 cells by electroporation along with plasmid DNA (2μg) for a beta galactosidase expression vector (pcDNA3 Beta gal). A and A', B and B' represent forward and reverse orientations respectively of the PCR fragments shown in fig.1. Cell

lysates were prepared 16 hours after transfection and assayed for luciferase and beta galactosidase enzyme activities. Luciferase enzyme activities are expressed relative to the luciferase activity obtained with the plasmid -40eIF luc transfected in the same experiment. Similar results were obtained in two independent experiments.

Fig.5: eIF4A1 directs high-level expression in macrophage cell lines. Panel A. Plasmid DNAs of the indicated plasmids (20μg) were introduced into RAW264.7 cells by electroporation along with plasmid DNA (2μg) for a beta galactosidase expression vector (pcDNA3 Beta-gal). Cell lysates were prepared 24 hours after transfection and assayed for luciferase and beta-galactosidase enzyme activities. Error bars represent the standard error of the means of two independent experiments.

Panel B. RAW cells were electroporated with the plasmid hCMVluc or –271eIF-IVS1 and the co-reporter plasmid pcDNA3 Beta-gal. Cells were harvested at 16, 24, 36, 48, 72 and 96 hours post transfection and cell lysates assayed for luciferase activity. Relative luciferase activities are given as a percentage of the value obtained at 16 hours normalised for beta-galactosidase activity at 16 hours. Similar results were obtained in two independent experiments.

Panel C. CHO cells were transfected with the plasmid hCMVluc or –271eIF-IVS1 and the co-reporter plasmid pcDNA3 Beta-gal. Cells were harvested at 16, 24, 40 and 48 hours post transfection and cell lysates assayed for luciferase activity. Relative luciferase activities are given as a percentage of the value obtained at 16 hours normalised for beta-galactosidase activity at 16 hours. Similar results were obtained in two independent experiments.

Fig.6: The plasmid –40 IVS1 (x1) contains one copy of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter fragment in pGL3Basic and plasmid –40 IVS1 (x2) contains two copies of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter. The plasmid pGL3Control contains the SV40 promoter and enhancer sequences cloned into the luciferase reporter plasmid pGL3Basic.

The indicated plasmid DNAs were introduced into the murine macrophage cell line RAW 264.7, human 293 cells and Chinese Hamster Ovary (CHO) cells along with a beta-galactosidase co-reporter plasmid (pcDNA3 Beta-gal). Cells were harvested 16 hours post transfection and cell lysates assayed for luciferase and beta-galactosidase enzyme activities. Normalised luciferase activities are expressed as fold induction compared to the promoterless vector pGL3Basic.

Fig.7 The eIF4A promoter and intron 1 are active in cancer cell lines. Luciferase reporter plasmids were introduced into (A) LNCaP (human prostate cancer) and (B) Cos-1 (African green monkey kidney), cells by electroporation and into (C) WiDr (human colorectal cancer) and (D) SKOV-3 (human ovarian cancer) using lipofectin. Cell lysates were prepared 48 hours post transfection and luciferase assays were performed and standardized by total protein content. All experiments were repeated in triplicate and showed similar values, but in each case a representative single experiment is shown.
(HSV1Tkmin = minimal thymidine kinase promoter from Herpes Simplex Virus 1).

Fig.8. The eIF4A promoter and intron 1 direct gene expression in vivo in mouse muscle. Luciferase reporter plasmids (50μg) were injected into the quadriceps muscle of C57Bl.6 mice. Muscles were removed after 72 hours and assayed for luciferase activity and the values standardized by protein content. Data is the mean of five animals. (-40EIF IVS1 A = one copy of intron 1, -40eIF4A IVS1 B = two copies of intron 1).

Fig.9:
DNA sequence of 5.318 kb upstream of the human eIF4A gene.
Figure 9

This sequence starts from the 5' Not I site of cosmid cos CD68C1, at –5280, where the transcriptional start site of the human eIF4A gene is +1, (Figure 2). It also includes the promoter and exon 1 sequence shown in Figure 2 and ends at the final base of exon 1, (+37). Regions of homology to Alu I repeat sequences or to the mouse eIF4A gene and surrounding sequences are underlined,(see Figure 1). The regions cloned and assayed for transcriptional actvity as PCR A (-

2102 to –1082) and PCR B (-1107 to –505) are highlighted in bold or italics, respectively, (see Figures 1 and 4).

Fig.10: Comparative luciferase activity of eIF4A and CMV promoter expression plasmids upon transient transfection into MK cells.

Fig. 11: Time course of promoter activity in MK cells.

Fig. 12: Efficacy of CMV and -271Eif-IVS1 promoters in particle mediated DNA delivery. Serum IgG response (1000 times dilution).

Fig. 13: Efficacy of CMV and -271eIF-IVS1 promoters in particle mediated DNA delivery. Serum IgG response 2 weeks post boost.

Materials and Methods

DNA sequence of the human eIF-4AI gene. Restriction enzyme fragments of the recombinant cosmid cosCD68C1 containing the human eIF4AI gene were subcloned into pBluescript SK-. DNA sequence was determined on both strands of the plasmid and cosmid templates using a Dye Terminator sequencing kit (ABI Perkin Elmer).

Reporter gene plasmids. Fragments of the human eIF4A1 promoter were PCR amplified to generate a 5' promoter deletion series using cosmid cosCD68C1 as template. Forward PCR primers included a Kpn I site and the reverse primer included a Hind III site, PCR products were digested with Kpn I and Hind III and cloned into the multiple cloning site of the luciferase reporter vector pGL3 Basic (Promega). Forward primers used were:
eIF–526 5' ATCTGGTACCCTACGATATCGCTGTTGATTTC (SEQ.I.D.NO:1),
eIF-371 5' ATCTGGTACCTGGAGGCTGAGACCTCGCC, (SEQ.I.D.NO:2)
eIF-271 5' ATCTGGTACCATGGCTGCCAGGCCTCGAGG, (SEQ.I.D.NO:3)
eIF-193 5' ATCTGGTACCGGCTGCGGGGCGGGCC, (SEQ.I.D.NO:4)
eIF-120 5' ATCTGGTACCTAGGAACTAACGTCATGCCG, (SEQ.I.D.NO:5)
eIF-98 5' ATCTGGTACCGTTGCTGAGCGCCGGCAGGC, (SEQ.I.D.NO:6)
eIF-69 5' ATCTGGTACCAAACCAATGCGATGGCCGG (SEQ.I.D.NO:7) and

eIF-40 5' ATCTGGTACCCGGGCGCTCTATAAGTTGTCG (SEQ.I.D.NO:8). The reverse primer used was
5' ATATAAGCTTTGATCCTTAGAAACTAGGGC (SEQ.I.D.NO:9).
Regions of the human eIF4AI 5' flanking region which show homology to the mouse eIF-4AI 5' flanking sequence were PCR amplified using the primers eIFBF 5' ATCTGGTACCGACTGGATTTCCACCAG (SEQ.I.D.NO:10) and eIFBR 5' ATCTGGTACCACCCAGGGCCACAGG (SEQ.I.D.NO:11). The region of non-homology with the murine eIF-4AI 5' flanking region was PCR amplified using the primers eIFAF 5' ATCTGGTACCTGTGGCCCTGGGTGG (SEQ.I.D.NO:12) and
eIFAR 5' ATCTGGTACCGGAAATCAACAGCGATATCGT (SEQ.I.D.NO:13). PCR products were digested with Kpn I and cloned in both orientations into the Kpn I site of eIF-40luc to give the plasmids BeIF-40luc, B'eIF-40luc, AeIF-40luc and A'eIF-40luc.
Introns of the eIF4A1 gene were PCR amplified with the following primers:
IVS1 F 5' ATCTAAGCTTCCCGGTAAGAAAGGCATTTG (SEQ.I.D.NO:13),
IVS1R 5' ATCTAAGCTTGGATCTGTTGGTTTAAAGCAT, (SEQ.I.D.NO:14)
IVS2F 5' ATCTAAGCTTGTCGACCCCGAAGGCGTCATCGAGGTGA (SEQ.I.D.NO:15),
IVS2R 5' ATCTAAGCTTGAATTCTAGGGGATGCAAAGA (SEQ.I.D.NO:16),
IVS3F 5' ATCTAAGCTTGTATCAAGGGTGAGACC (SEQ.I.D.NO:17),
IVS3R 5' ATCTAAGCTTCATAACCTAAACAAATAAATT (SEQ.I.D.NO:18),
IVS4F 5' ATCTAAGCTTCTCAGCAGGTAAGAGTGG (SEQ.I.D.NO:19) ,
IVS4R 5' ATCTAAGCTTGAATTCCCTTCTGTATCTGAGCAG (SEQ.I.D.NO:20) ,
IVS5F 5' ATCTAAGCTTTGCTGGTTTCTCTCTGG (SEQ.I.D.NO:21),
IVS5R 5' ATCTAAGCTTGAATTCGGGCTAGAGAAGAAAAA (SEQ.I.D.NO:22),
IVS6F 5' ATCTAAGCTTCCCAGGTGAGGGCAGT (SEQ.I.D.NO:23) ,
IVS6R 5' ATCTAAGCTTGAATTCAGCAAAACTACCTAGTGGA (SEQ.I.D.NO:24),
IVS7F 5' ATCTAAGCTTCGTGGAACGAGAGGTGG (SEQ.I.D.NO:25),
IVS7R 5' ATCTAAGCTTGAATTCCTTCCACTCCTGGAGGTT (SEQ.I.D.NO:26),
IVS8F 5' ATCTAAGCTTTGGTGTGTTTGCCCCCT (SEQ.I.D.NO:27),
IVS8R 5' ATCTAAGCTTGAATTCTGCTGGAAGAGAAAACAAA (SEQ.I.D.NO:28),
IVS9F 5' ATCTAAGCTTCTGACCTGCTGGTGAGTAG (SEQ.I.D.NO:29) and

IVS9R 5' ATCTAAGCTTGCCTCTGGCCTACGTCAAGAAAG (SEQ.I.D.NO:30). PCR fragments were digested with HindIII and cloned into the unique HindIII site of plasmid eIF-40luc, which lies 3' of the transcription initiation site of the eIF4AI gene promoter. Restriction mapping and DNA sequencing was used to identify plasmids containing introns in the correct orientation. Introns 1, 2 and 3 were excised with Hind III, rendered blunt ended by treatment with Klenow DNA polymerase and cloned into the KpnI site of –40eIFluc to give the plasmids IVS1eIF-40luc, IVS2eIF-40luc and IVS3eIF-40luc in which the introns are placed 5' of the eIF4A1 minimal promoter. The plasmid pGL3 Control contains 195bp of the SV40 promoter cloned 5' of the luciferase reporter gene and 244bp of the SV40 enhancer cloned 3' of the luciferase reporter gene (Promega). The plasmid hCMVluc was constructed by cloning the Nru I / Hind III CMV promoter fragment from pcDNA3 (InVitrogen) into the Sma I / Hind III polylinker sites of pGL3 Basic (Promega). The 200bp Herpes Simplex Virus 1 thymidine kinase promoter was PCR amplified and cloned into KpnI/Bg II sites of pGL3 Basic (Promega). The plasmid pcDNA3 Beta gal (14) contains the E.coli lacZ gene cloned into the mammalian expression vector pcDNA3 (InVitrogen). Plasmid DNA for transfection was prepared from 500ml cultures of E.coli grown overnight in LB broth containing 100μg ampicillin (Sigma) using a standard NaOH/SDS lysis protocol and centrifugation in CsCl ethidium bromide gradients (12).

Mammalian cell culture and transient transfection. The murine macrophage cell lines RAW264.7 and P388.D1, the murine B cell line A20 and the human erythroleukaemic cell line K562 and the human prostate cancer cell line, LNCaP were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat inactivated foetal calf serum (FCS) (Sigma, St. Louis, MO), 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 2mM glutamine. CHO K1 and SKOV-3 cells were maintained in Ham's F-12 medium and 293 cells WiDr and COS-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, antibiotics and glutamine. All cells were grown at 37°C in a humidified incubator in 5% CO_2/air mixture.

RAW264.7, P388.D1, A20 and K562 LNCaP and COS-1 cells were transfected by electroporation. Briefly, cells were grown to confluence in T175 flasks,

harvested in Phosphate Buffered Saline (PBS), washed once with PBS and resuspended in Optimem 1 serum free medium (Life Technologies) for RAW cells or RPMI 1640 (no FCS). Aliquots of 2 x 10^7 cells (0.5ml) were mixed with 20μg luciferase reporter plasmid DNA and 2μg pcDNA3 β-galactosidase plasmid DNA, added to a 0.4cm electrode gap electroporation cuvette (BioRad, Hercules, CA) and shocked in a BioRad GenePulser (300V, 960μFD) at room temperature. Cells were recovered immediately into 10ml of pre-warmed medium and plated into 9cm diameter tissue culture petri dishes (Nunc).

CHO and 293 cells were grown to 70-80% confluence in 9cm petri dishes, washed twice with Optimem before addition of 5ml of plasmid DNA:cationic lipid complex (5μg DNA: 50μg Lipofectamine (Life Technologies) in Optimem). After 4-6 hours incubation the medium was aspirated, cells were washed twice with PBS and recovered into complete medium for 16 hours before analysis. SKOV-3 and WiDr cells were similarly transfected using 5μg DNA, 50μl lipofectin (Life Technologies)

Reporter gene assays Transfected cells were harvested in 500μl Reporter Lysis Buffer (Promega) and lysed with one cycle of freeze thaw. Cell lysates were assayed for β-galactosidase enzyme activity using the colorimetric substrate chlorphenolred β-D-galactopyranoside (CPRG, Boehringer Mannheim) in a 96 well plate assay in 50mM potassium phosphate buffer (pH7.3) with 2mM $MgCl_2$. Enzyme activity was determined by spectrophotometry at 570nm after 30 minutes incubation at 37°C using dilutions of purified E.coli β-galactosidase enzyme (Sigma) to generate a standard curve. Luciferase enzyme activity was determined using a Luciferase Assay Kit (Promega) and a Berthold Instruments LB9501 luminometer. All luciferase enzyme assays using transfected cell extracts were within the linear range of the assay.

In vivo luciferase assays.

Luciferase reporter plasmids, (50ug), were injected into the quadriceps muscles of C57Bl/6 mice. Muscles were removed after 72 hours and disrupted in 500ul of reporter lysis buffer, (Promega), using an IKA Labortechnic Ultra turrax T8 polytron. Luciferase enzyme activity was determined using a luciferase assay kit, (Promega) and a Dynatech Laboratories ML3000 luminometer. Total protein content was measured in a 96 well format using a Pierce Coomassie Plus

Protein Assay Reagent against a standard curve using a Molecular Devices Spectra Max 250 plate reader.

Results

eIF4AI gene organisation

We have shown previously that the macrophage restricted CD68 gene lies 669bp 3' of the gene encoding the ubiquitously expressed translation initiation factor eIF-4AI gene on chromosome 17p13 (13,14). We determined the DNA sequence of 11.9kb of CD68 5' flanking sequence and showed that the human eIF4A1 gene is 6.2kb in size and contains ten introns shown schematically in Fig.1. The position of the eIF4A1 gene introns and their sizes are listed in Table 1.

Intron	Size	Sequence
Intron 1	1397 bp	AGGATTCCCGgtaagaaagg...aaaccaacagATCCAGAGAC
Intron 2	226 bp	CGTCATCGAGgtgagactgg...catcccctagAGTAACTGGA
Intron 3	440 bp	TGTATCAAGGgtgagacctc...atttgtttagGTTATGATGT
Intron 4	1264 bp	GGCTCAGCAGgtaagagtgg...ctctgctcagATACAGAAGG
Intron 5	363 bp	AGATACCTGTgtgagtaatt...tcttctctagCCCCCAAATA
Intron 6	179 bp	CAACACCCAGgtgagggcag...tttccactagGTAGTTTTGC
Intron 7	81 bp	GGAACGAGAGgtggggccca...caacctccagGAGTGGAAGC
Intron 8	120 bp	ATCCGCCATGgtgtgtttgc...tctcttccagCATGGAGATA
Intron 9	248 bp	TGACCTGCTGgtgagtagag...cttgacgtagGCCAGAGGCA
Intron 10	96 bp	ATATCCACAGgtaagcgtag...tgttttccagAATCGGTCGA

Table 1: eIF4A1 intron sizes are given in base pairs. The exon/ intron boundary sequences are shown with the exon sequences in upper case and the intron sequences in lower case.

The first intron of the eIF4A1 gene contains many CpG dinucleotides and has many features of a methylation free island. An analysis of the first intron DNA sequence revealed the presence of many potential binding sites for mammalian transcription factors. eIF4A1 intron 1 contains three consensus binding sites for

Sp1, two C/EBPβ sites, two binding sites for each of the ets factors Elk-1 and c-ets-1 and 8 binding sites for the transcription factor GATA-1. The 440 bp third intron of the eIF4A1 gene contains a 35bp region containing four overlapping consensus binding sites for GATA 1 and three for GATA2 (Fig. 2 and data not shown).

eIF4A1 promoter sequence

Comparison of the DNA sequence of the human eIF4A1 gene 5' flanking region with the published sequence of the murine eIF-4AI gene sequence (15) revealed a 300bp region showing ~85% sequence homology with the mouse eIF-4AI gene proximal promoter. This region contains a canonical TATA box sequence at position –32 relative to the putative transcription start site (16), CCAAT box sequences at positions –65, -141 and –190 and consensus binding sites for the transcription factor Sp1 at positions –51, -74, -177 and -348, (Fig. 2). Homology with the murine eIF-4AI promoter sequence ends at position –475 but homology with the murine promoter continues at position –1110 and extends for a further 254 bp to the end of the published murine eIF-4AI sequence. The 638 bp region of non-homology with the murine promoter (positions –1109 to –471) contains an extended dinucleotide repeat sequence $(AT)_{38}$, alternating AT repeats have been shown to be present as interspersed repetitive elements in several eukaryotic genomes (17). Analysis of the eIF4A1 5' flanking sequence revealed the presence of AluI interspersed repeats at positions –4588 and –3722 relative to the eIF4A1 transcription start site (Fig.1).

eIF4AI promoter analysis

A 5' deletion series of the eIF4A1 gene promoter was constructed in the luciferase vector pGL3 Basic and plasmid DNAs were introduced into a range of mammalian cell lines by transient transfection. A DNA fragment extending from –526 to + 15 of the eIF4A1 gene directs high-level luciferase reporter gene expression in the epithelial cell lines CHO and 293 and in the murine macrophage cell line RAW 264.7 (Fig. 3A-C). In CHO cells the maximal level of luciferase expression is obtained with plasmid –526eIFluc and the level of expression is similar to that obtained with the SV40 enhancer/promoter vector pGL3 Control (Fig. 3A). In 293 cells the maximal level of luciferase expression is obtained with the –371eIFluc plasmid with the level of luciferase expression

being 18 times that obtained with the SV40 enhancer/promoter (Fig. 3B). The –40eIFluc plasmid which contains only the TATA box and the –69eIFluc plasmid which contains a CCAAT box, an Sp1 site and the TATA box give levels of reporter gene expression only slightly higher than that seen with the reporter gene vector alone (pGL3 Basic). The –98eIFluc plasmid which contains an additional 29bp of eIF4A1 promoter sequences contains an AP-2 site and directs high-level gene expression in all three cell lines tested. The –120eIFluc plasmid directs similar levels of reporter gene expression in 293 cells but significantly reduced levels of reporter gene expression are seen in CHO and RAW 264.7 cells. Addition of eIF4A1 sequences 5' of –120 restores high levels of reporter gene expression.

PCR primers were designed to amplify human eIF4A1 gene sequences 5' of position –1110 that show homology with the murine eIF-4AI promoter. A 1018 bp fragment containing eIF4A1 gene sequences extending from position –2098 to –1080 was cloned in both orientations 5' of the eIF4A1 minimal promoter in the reporter gene vector –40eIFluc to give the plasmids BeIF-40luc and B'eIF-40luc. A 597bp region of the human eIF4A1 promoter that shows no homology with the murine eIF-4AI promoter was cloned into the reporter gene vector –40eIFluc to give the plasmids AeIF-40luc and A'eIF-40luc. In CHO cells the plasmids BeIF-40luc and B'eIF-40luc give levels of luciferase expression only twofold higher than that obtained using the eIF4A minimal promoter (Fig. 4A). In 293 cells the same plasmids enhance expression between 4 and 6-fold (Fig. 4B) while in RAW cells expression is enhanced 5-fold (data not shown). By contrast the region of non-homology with the murine eIF-4AI promoter present in plasmids AeIF-40luc and A'eIF-40luc enhances expression up to 27-fold in CHO cells, 13-fold in 293 cells and 5-fold in RAW cells (Fig 4).

Analysis of transcriptional regulatory elements in eIF4AI gene introns

eIF4A1 intron 1 contains a methylation free island and potential binding sites for several ubiquitous transcription factors such as Sp1, c-ets1 and Elk-1. Potential transcription factor binding sites are found in other introns of the eIF4A1 gene. We prepared a series of luciferase reporter plasmids in which the first nine introns of the eIF4A1 gene were cloned downstream of an eIF4A1 minimal promoter fragment and tested their activity in transient transfection assays. The

data are summarised in Table 2 showing the fold increase in reporter gene activity compared to the eIF4A1 minimal promoter alone (plasmid -40eIFluc).

Plasmid	K562	A20	CHO	293	P388.D1
-40eIF luc	1	1	1	1	1
-40eIF IVS1	260	52.6	29.8	51.6	482
-40eIF IVS2	10.7	7.2	2.2	19.8	5.7
-40eIF IVS3	20	12.6	3.7	26.4	4.3
-40eIF IVS4	0.4	1.9	0.6	0.8	0.6
-40eIF IVS5	7.3	9.5	5.3	14.2	2.9
-40eIF IVS6	10	5.2	4.5	14.3	2.6
-40eIF IVS7	25.3	14.7	5.5	23	6.3
-40eIF IVS8	0.1	0.1	n.d.	n.d.	0.1
-40eIF IVS9	18	8.7	3.3	9.6	2.2

Table 2 Effect of eIF4A1 introns on transcription from an eIF4A1 minimal promoter.

With the exception of introns 4 and 8 seven of nine eIF4Al introns tested were shown to enhance expression when cloned downstream of the eIF4A1 minimal promoter. In all cell lines tested the greatest enhancement of expression is seen when the 1397bp first intron of the eIF4A1 gene is cloned downstream of the eIF4A1 minimal promoter. The magnitude of the observed enhancement varies from 30-fold in CHO cells to 480-fold in the murine macrophage cell line P388.D1. Introns 2, 3, 7 and 9 were able to enhance reporter gene expression more than 15-fold. However, the activity of these introns varied widely between different cell lines, for example, the 83bp eIF4A1 intron 7 enhances luciferase expression 25-fold in K562 cells but only 6-fold in CHO and P388.D1 cells.

To test if intron 1 of the eIF4A1 is able to act as a classical transcriptional enhancer a 1397bp IVS1 fragment was cloned 5' of the eIF4A1 minimal promoter in the plasmid -40eIFluc to give the plasmid IVS1eIF-40luc. Reporter gene plasmids with eIF4A1 placed 5' or 3' of the eIF4A1 minimal promoter were transfected into the murine macrophage cell line RAW 264.7. Placing eIF4A1 IVS1 3' of the eIF4A1 -40 promoter fragment enhances luciferase expression

1700-fold while placing IVS1 5' of the eIF4A1 -40 promoter enhances expression 680-fold (Table 3). Thus, eIF4A1 intron 1 sequences are able to enhance gene expression when placed 5' or 3' of the transcription start site.

	-40eIF luc	-40eIFIVS1	IVS1-40eIF	pGL3 Basic
RLU/ Beta galactosidase (x1000)	60	102000	41000	72
Fold –40eIF luc	1 x	1700 x	683 x	1.2 x

Table 3: The indicated luciferase reporter plasmids (20μg) were electroporated into RAW 264.7 cells with the beta galactosidase reporter plasmid pcDNA3 Beta gal (2μg). Cell lysates were prepared 24 hours post transfection and assayed for luciferase and beta galactosidase enzyme activities. The results shown are representative of two independent experiments.

Sustained high-level reporter gene expression in macrophages using eIF4A1 sequences

In order to develop an expression vector using eIF4A1 sequences we cloned eIF4A1 intron 1 downstream of selected eIF4A1 promoter fragments and compared the level of luciferase expression obtained with eIF4A1 sequences with SV40 and CMV enhancer/promoter sequences cloned into the same reporter gene vector. Addition of the eIF4A1 intron 1 sequence increases reporter gene expression in RAW cells from all the eIF4A1 promoter fragments tested. The level of luciferase activity obtained with the plasmid -271eIFIVS1 is equal to that obtained with the human cytomegalovirus promoter/enhancer in the plasmid hCMVluc and more than three times that obtained with the SV40 enhancer/promoter in the plasmid pGL3 Control (Fig. 5).

The data of Figure 5A were obtained with RAW cells harvested 24 hours post transfection. RAW cells were transfected with CMV and eIF4A1 promoter containing plasmids and a comparison of the level of reporter gene expression over a 96 hour period is shown in Figure 5B. The levels of CMV-driven reporter gene expression in RAW cells declines to less than 10% of initial levels 48 hours post transfection. The level of reporter gene expression obtained in RAW cells

with the –271 eIF4A1 promoter fragment and eIF4A1 intron 1 is ten fold greater than that seen using the CMV promoter/enhancer 72 hours after transfection (Figure 5B). The same experiment was performed in non-macrophage cell lines and the data of Figure 5C show a comparison of CMV- and eIF4A1-driven reporter gene expression in RAW cells. The rapid decline in CMV-driven expression seen in RAW cells is not seen in CHO cells. However the –271eIF-IVS1 plasmid gives sustained high-level reporter gene expression in CHO cells with a five fold increase in luciferase expression between 16 and 48 hours post transfection (Figure 5C).

Expression characteristics in murine macrophages using more than one eIF4A1 intron.

The plasmid –40 IVS1 (x1) contains one copy of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter fragment in pGL3Basic and plasmid –40 IVS1 (x2) contains two copies of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter. The plasmid pGL3Control contains the SV40 promoter and enhancer sequences cloned into the luciferase reporter plasmid pGL3Basic.

The indicated plasmid DNAs were introduced into the murine macrophage cell line RAW 264.7, human 293 cells and Chinese Hamster Ovary (CHO) cells along with a beta-galactosidase co-reporter plasmid (pcDNA3 Beta-gal). Cells were harvested 16 hours post transfection and cell lysates assayed for luciferase and beta-galactosidase enzyme activities. Normalised luciferase activities are expressed as fold induction compared to the promoterless vector pGL3Basic (see fig.6). Cloning two copies of eIF4A1 intron 1 downstream of the eIF4A1 –40 promoter gives an increase in reporter gene expression compared to the level obtained using one copy of intron 1 in all three cell lines tested. The additional increase in reporter gene expression is small in RAW cells (10%) but the second copy of eIF4A1 increases gene expression in 293 cells more than five fold.

The eIF4A promoter and intron 1 direct gene expression in vivo in mouse muscle. In vivo eIF4A promoter / intron activity has been determined in mouse muscle by injection of luciferase reporter plasmids, (Fig. 8). The -526 fragment

plus intron- 1 shows over three times the activity of the SV40 promoter / enhancer combination in this system.

The eIF4A promoter and intron 1 are active in cancer cell lines. Plasmids where luciferase expression is driven by the eIF4A promoter fragment and intron 1 (IVS-1) regions were introduced into a series of cancer cell lines: LNCaP, COS-1, WiDr and SKOV-3, (Figure 7).. Normalised luciferase activities show that the eIF4A promoter and IVS-1 are active in all cell types tested. The -271 and -526 promoter fragments are most active as in other cell types, (see Figs. 3 & 5). For example, the -526 and IVS-1 combination drives almost two fold more luciferase expression than the SV40 promoter / enhancer combination in WiDr, colorectal cancer cells and over 500 fold more expression in LNCaP, prostate cancer cell line.

By combining eIF4A1 promoter sequences with eIF4A1 intron 1 we were able to direct sustained high-level reporter gene expression in macrophages. The levels of luciferase gene expression obtained in murine macrophages using eIF4A1 vectors were three times higher than that seen with the SV40 promoter/enhancer and equal to the level of expression seen using the human CMV promoter/enhancer.

Comparison of transcriptional activity of -271eIF4A and CMV promoters in murine keratinocytes.

The inventors have also shown that the eIF4A promoter is more active in murine keratinocytes than the CMV promoter. Again this demonstrates in a cell line other than an antigen presenting cell that the eIf4A promoter is stronger than the CMV promoter. Also murine keratinocytes provide a cell line model for the cell type other than professional APCs that could contribute to the antibody response via the gene gun by expressing antigen *in vivo*. This example shows a sustained high-level reporter gene expression in keratinocytes using eIF4A1 sequences.

Mammalian cell culture.

The murine keratinocyte cell line , MK, (Weissman, B.E. and Aaronson, S. A., 1983, Cell **32**, 599-606), was maintained in S-MEM, (Spinner Culture Modified

Eagle's Medium) , Life Technologies supplemented with 10% foetal calf serum , Life Technologies, 100 units/ml penicillin, 100ug/ml streptomycin, 2mM glutamine and 4ng/ml human recombinant EGF, (Sigma). Cells were grown at 37°C in a humidified incubator in 5% CO_2/air mixture.

Transient transfections.

MK cells were grown to 80% confluence in 6 well plates, washed twice with 1ml Optimem and transfected with 1ml of plasmid DNA: cationic lipid complex, (5ug DNA: 30ul Transfast ™, Promega), in Optimem. Cells were generally harvested 48 hours post transfection for luciferase assays or at specific time points for a time course of activity.

Luciferase assays.

For luciferase assays, transfected cells were harvested in 1ml Passive Lysis Buffer and luciferase activity was determined using a luciferase assay kit, (Promega) and a ML3000 plate luminometer, (Dynatech Laboratories). Total protein content was measured in a 96 well format using Pierce Coomassie Plus Protein Assay Reagent against a standard curve using a Spectra Max 250 plate reader, (Molecular Devices).

Level of transcriptional activity

The level of transcriptional activity of eIF4A1 promoter fragments combined with intron 1, (IVS-1) compared to that of the CMV promoter in murine keratinocytes, was determined. Plasmids containing the relevant promoter / intron combinations driving luciferase expression were transfected into MK cells. Luciferase activity was assayed 48 hrs post-transfection and expressed, (RLU / mg protein), as a percentage of the CMV luc plasmid, (set as 100%). The relative luciferase activity , (Fig 10), indicates that the plasmid –271eIFIVS1 directs about three times as much luciferase expression as the plasmid hCMVluc. Figure 10 represents a mean of 4 separate experiments.

Time course of promoter activity

MK cells were transfected with the plasmid hCMVluc or –271eIF-IVS1luc. Cells were harvested at 22, 31 and 48 hours post transfection and assayed for luciferase actvity and total protein. Relative luciferase activities, (RLU/ mg

protein), are given as a percentage of the value from the first time point for each plasmid transfected. Figure 11 is a mean of 2 experiments and shows that the -271eIF-IVS1 plasmid gives sustained high-level reporter gene expression in MK cells with about a six fold increase in luciferase expression between 22 and 48 hours post transfection. Note that the rapid decline in CMV driven expression seen in RAW cells, (Fig. 5A), is not seen in MK cells.

Efficacy of –271eIF-IVS1 promoter in DNA vaccination

The following example shows that when the eIF4A promoter is driving expression of influenza nucleoprotein (NP) antigen in a particle mediated DNA delivery experiment, it produces as good or a better antibody response than an equivalent plasmid with CMV driven NP expression.

Cartridge preparation

Preparation of cartridges for particle-mediated DNA delivery (PMDD) using the Accell gene transfer device was as previously described (Eisenbraun et al DNA and Cell Biology, 1993 Vol 12 No 9 pp 791-797). Plasmid DNA was coated onto 2 □m gold particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 4□C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with between 0.5 – 0.8 □g plasmid DNA.

Animals and immunisations

Female C57Bl/6 mice, purchased from Harlan UK Ltd (Oxon, United Kingdom) or bred in our specific pathogen-free animal breeding facilities, were housed at a constant temperature and humidity, with regular 12 hour cycles of light and darkness, sterile bedding, food and water. All experiments were carried out under United Kingdom ethical guidelines. Mice were immunised between 6-8 week of age. For each immunisation, plasmid DNA was delivered to the shaved target site of abdominal skin by PMDD from two cartridges using the Accell gene transfer device at 500 lb/in^2 (McCabe WO 95/19799), delivering a total of between 1.0 -1.6 μg DNA per immunisation. The DNA vaccination regime included a primary immunisation followed by one boost 6 weeks later.

tion for antibody ELISA assays

s were collected from the tail vein 1-3 days before immunisation, after primary and 15 days after boost immunisation. Serum was red at -20°C for subsequent antibody analyses.

d recombinant nucleoprotein (NP) of influenza virus as a se fusion protein for serum antibody ELISA assays tein of influenza A virus strain PR/8/34 was cloned as modified pUC18 vector. This was then sub-cloned as EX-4T-3 (Pharmacia) and DNA sequence analysis would express an in-frame fusion with GST.

transformed with pGEX flu/NP were grown to otein expression was induced by the addition ide to a final concentration of 0.1mM and urs at 37°C. Cells were harvested by s and the pellet resuspended in 30ml of d by sonication at maximum amplitude s were removed by centrifugation at was removed and incubated with 1h at 4°C with gentle agitation. rifugation and washed 3 times eluted by incubation of the ced glutathione (Sigma) in ure. The purified protein utathione and stored at

nologies) were
shed 4 times
ium azide).
5% BSA
s we-

buffer. After 4 further washes (as above) to remove unbound antibody, plates were incubated for 1 hour with peroxidase conjugated anti-mouse IgG antibodies (Southern Biotechnology, Birmingham, AL, USA) diluted in blocking buffer. The amount of bound antibody was determined after 4 further washes (as above) followed by addition of TMB substrate solution (T-8540, Sigma). After 30 minutes at 20°C and protected from light the reaction was stopped with 1M sulphuric acid and absorbance read at 450 nm. Titres were defined as the highest dilution to reach an OD of 0.2.

Comparison of in vivo effect of -271eIF4A and CMV promoters on IgG antibody response to pVAC1.PR

The NP-specific serum IgG levels were measured before immunisation, and at intervals following the primary and boost immunisations. The construct with the -271eIF4A promoter gave similar responses to those following immunisation with the construct with the CMV promoter (Fig. 12), with equivalent titres for both promoters after the boost immunisation (Fig. 13).

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SEQ.I.D.NO:31

GTAAGA AAGGCATTTG CAAGAGATTG TGGCTGCTTA

TTTTGCCGCC CCCTTCCGAC GGGCCCGCCG GGGGTAGCTG AGAGGCCCAC

CAGGGTTGCG GGAGAAACCG AACCGGGTGG GGGGAGGGTC CGACTTGGAG

GGGCGAGGGG GAAGACCCAC GGCCGACGCG GCCACCAGGT CGAGGCGGAG

GGTAGGGACA GCCCGGCTAG GGTCAGGCGT GCGAGGTCTG TTACGAGGCC

TCGACCCGAG GCGGTGCCAT GCGCGAAGCC CCGGCGCTGA GTGGCGAGAC

GGGGTCGCGA CCTGGCGTGG GAAAGAAAGG TGGAGGCGGC CGCCACTATG

TGTGGCCCAG AGCCGGCAGG TCCGGTTGCC TCCCTGTGCC GGGGGAGGGA

CGGCGCGCGG GGTTCCGGAG CATTCTGACG GTACCACTCG CGAGAGGCGG

GGGTGCCTGG TCCTTAGATC CAGTCACTTC GTCGCGGCTA AAACACGGGT

CGGGGAGAAG AAACCGGCCG TTCAGTGTGC TGGTTTTCTT GACGGCCAGG

ACTGAGCCTA ACCCCGAGG. AGCGGCCGCG TGAGGCACCA GGAGCCCACC

CGGCGCCGGG CGGGCGGGTC CATTTTGCCG CACAAGCCGG GCTATTGGCA

AACTGCGGAT GGGCAGGTCC ACCTTCCTTC GGGGGTGAGC GGCCTGAGGT

ATGGGAGGGC GACGCTACTT CGCGACGGGG GCGGGCGGGA TGTGGATTGT

TCCATGGAGG GGTGGGAGAC RCCGCCGGGT GGTCGARGGA GCGAGCACAT

GGTGGCCTGA GGCGTTCCCC TCCCCCAGTC TGCTTCGCTT CTAAGTGTTG

TGCAATCTCC CCCTTTGCTA GCTCGGCTTG GGCTCATTGT GCGCGAGGCC

GCCACCGCCC GCGGCCTCCC ACATCCGGGC AACGCGAGGG GGGGGCTTCG

GCTGGAGGGA GTGGGGGAGG GCGCGGGCGG GATGACGTGG GGGGAAGGGG

ATGTCCTACC CTCCGATCTG GGAGGTGAAG GGCGGGACTT CCAGCGCGCT

GGTGCTGCGG TGGGAGGTGC ACGCGCTTGG GCTTTAAGCG GCTGGGTCGG

GCCCACGTGG ACCCGGCGGC AAGCACCACC TNTGGGCACC GTGAGCGCGG

CGGCACGCCT GCCGGCCTGT CTTCAGAAAG GGTCACCCCC TTATGTCGGG

GGTGGCCTGG CCTGAGCCGC TGCCTGCATG GGGCAAATGC CTCAGTTTTA

TAGAAACTCC TCCTTTGGGT ATTTTTTGGG AGCTGGTGGG AGTTGGATCT

GGGACAGCAG GTTGATGGCA TCATGCAGGC CACTCCTGAC AGAGCCCGGC

TGTCAGGATT TCTGAGTGCT TCGGTCGGGC AGGGGACAAA ACTTATGCTT

TAAACCAACA G

SEQ.I.D.NO:32

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GTATAGAGTT AGAGTGGCCT CTTGATTGAT TTCCCAGATC ATCTAGAAGC

AGCTGGTTTC CCTAAAGGGA GGAGGGTTGT AAGCTCTGAG GCTTTTGTTA

RTASGCACCA SATTCTGTTT GCTCGGAGAC TACAGCTCAG CTCCACCTTT

TCCATGACTC AAGCTTTAAT TTCTTTGCAT CCCCTAG

SEQ.I.D.NO:33

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SEQ.I.D.NO:34

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CCT GGG TCA CTT TGC TCT TGT GCA
CGC TTT CCA GTC TTT CAG CGT AAG
CCA GAG TCA TTC CCA AGG ATG CTG
GTT TCT CTC TGG GGG AAG AGC TGC
TCT GTG ATG GAG CCC ATG CGT GTC
ATC TGA GCC TCT GGC TTC CCT GCC
AGT GCA GCC CTG GCA GTG TCC TAC
TTC CCA GGG CTG TTG TCT GCC TGG
CGG GAA GGT CCT GGG CAA AGG ATC
AGT CTT

TGTACTCTGA GAGCAGACTA
CTTGGCTCCT CTCTGTTTTT
TATCAGCGAA

GTTGGATATA TCTCTCCCAC
ATTTCCCTAA TCATATGCTA
TATATTGGCT

TTTTTTTTCT TCTCTAG

SEQ.I.D.NO:35

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AAA TAG TAA GTG CCA GGG GAA ACC AAA TAC TGG ATT CTT GAG CCT TTT TAT GCA TCT GCT TCA GTT TTA GGT GTG GCT AGG GAA GGG AGC AGG CCT CAG GAA GGA ACC AGC ACT CTA AGA CTG GCC TTT TTT TCC ACT AG

SEQ.I.D.NO:36

GTG GGG CCC AGT GCA GGA GGC GGG CCT GGT AGT GAG TTG TTG GGT ATA GCC CCT GAC TGA TTT TTG TCC CCC AAC CTC CAG

SEQ.I.D.NO:37

GTG AGT AGA GGG AAC TGA TAG CAA AGG CAG AAG GGA GGA TCC AAG GTG ATT CCC TCT CCA AGG GGA CAT CAG TGC CTC TCA GGA AAG TAG C

AGCTTGGAAT AGAATCTGGC ATGCCTAAGG CCTTTGGGGA ACTGGGATGC

TTATTTCCTC TGCCTTCCTT GGCTGCCCAC ATGGATGCCT AAGTGTCTTC

CCTCCGGGAT AGAGTGTCCT CCGTGCACAT GCTGAAGAGT TGTCTTTCTT

GACGTAG

SEQ.I.D.NO:38

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ACA CGT CCA GGC AGT GTC GAA TCC ATC TCT GCT ACA
GGG GAA AAA CAA ATA ACA TTT GAG TCC AGT GGA GAC
CGG GAG CAG AAG TAA AGG GAA GTG ATA ACC CCC AGA
GCC CGG AAG CCT CTG GAG GCT GAG ACC TCG CCC CCC
TTG CGT GAT AGG GCC TAC GGA GCC ACA TGA CCA AGG
CAC TGT CGC CTC CGC ACG TGT GAG AGT GCA GGG CCC
CAA GAT GGC TGC CAG GCC TCG AGG CCT GAC TCT TCT
ATG TCA CTT CCG TAC CGG CGA GAA AGG CGG GCC CTC
CAG CCA ATG AGG CTG CGG GGC GGG CCT TCA CCT TGA
TAG GCA CTC GAG TTA TCC AAT GGT GCC TGC GGG CCG
ATG TCT GCG AGC CAG GAT TCC CG

Claims

1. A DNA construct comprising a transcriptional regulatory sequence operatively linked to a heterologous gene of interest which sequence comprises a polynucleotide derivable from the eIF4A1 gene promoter or fragment thereof.
2. A DNA construct comprising a transcriptional regulatory sequence operatively linked to a gene of interest which regulatory sequence comprises a polynucleotide derivable from the eIF4A1 gene promoter or fragment thereof and at least one sequence derivable from a eIF4A1 gene intron.
3. A DNA construct comprising a transcriptional regulatory sequence operatively linked to a gene of interest which regulatory sequence comprises a polynucleotide derivable from the eIF4A1 gene promoter or fragment thereof and at least one intron sequence of the eIF4A1 gene wherein the intron sequence is selected from the group consisting of; intron 1, 2,3,5,6,7,9.
4. A construct according to any preceding claim wherein the eIF4A1 gene is mammalian.
5. A construct according to claim 2 or 3 wherein the intron sequence of the eIF4A1 gene is mammalian.
6. A construct according to any one of claims 2 to 5 wherein the intron sequence is intron 1.
7. A construct according to any one of claims 2 to 6 wherein the transcriptional regulatory sequence comprises a polynucleotide derivable from a plurality of eIF4A1 intron sequences.
8. A construct according to any preceding claim wherein the eIF4A1 gene promoter fragment is selected from the group consisting of; -526EIF, -371EIF, -271EIF, -193EIF, -120EIF, -98EIF, -69EIF and -40EIF.
9. A construct according to any preceding claim wherein the transcriptional regulatory sequence comprises a polynucleotide derivable from a variant of the eIF4A1 gene promoter which variant results from an addition, insertion, substitution, deletion,

rearrangement or modification of one or more bases of the eIF4A1 gene promoter.

10. A construct according to any preceding claim wherein the construct is a phage, plasmid, viral, minichromosome or transposon, preferably plasmid.
11. A construct according to any one of claims 2 to 10 wherein the eIF4A intron gene sequence is located downstream (in terms of reading frame) from the eIF4A gene promoter.
12. A construct according to any preceding claim further comprising one or more of the following; a terminator sequence, a bacterial origin of replication, a selectable marker.
13. A construct according to any preceding claim wherein the gene of interest encodes a therapeutically useful protein such as interferon-β, factor VIII, erythropoetin, growth factor or cytokine.
14. Host cell comprising (harbouring) a construct according to any preceding claim.
15. A host cell according to claim 14 wherein the cell is eurkaryotic, preferably mammalian.
16. A process for the production of a protein which comprises the step of culturing a host cell according to claim 14 or 15 and optionally recovering the protein.
17. A method of treating a disease or disorder characterised by clinically significant cell loss (particularly neuronal cell loss) comprising the step of administrating a therapeutically effective amount of the host cell according to claims 14 or 15.
18. A method according to claim 17 wherein the disease or disorder is Parkinsons.
19. A method of correcting or compensating for a disease or disorder whose etiology is characterised by a genetic abberation such as cystic fibrosis which method comprises the step of administrating to a mammalian patient a therapeutically effective amount of the construct of any one of claims 1 to 13.
20. A medicament, particularly a pharmaceutical composition, comprising a construct according to any one of claims 1 to 13.

21. A medicament according to claim 20 wherein the construct is incorporated into a carrier such as a (cationic) liposome.
22. A method of treating a disease or disorder comprising the step of administrating a therapeutically effective amount of the medicament according to claim 20 or 21.
23. A DNA construct comprising a transcriptional regulatory sequence operatively linked to a heterologous gene of interest which regulatory sequence comprises a polynucleotide having a sequence as set forth in SEQ.I.D.NO:38 or variant thereof.
24. A DNA construct according to claim 23 wherein the regulatory sequence further comprises one or more of the sequences as set forth in SEQ.I.D. NO: 31 to 37.
25. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:31 or variant thereof.
26. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:32 or variant thereof.
27. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:33 or variant thereof.
28. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:34 or variant thereof
29. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:35 or variant thereof.
30. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:36 or variant thereof
31. An isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:37 or variant thereof.
32. An isolated polynucleotide having a sequence as set forth in fig.9 at positions -2102 and -1082 or variant thereof.
33. An isolated polynucleotide having a sequence as set forth in fig. 9 at positions –1107 to –505 or variant thereof.
34. A construct according to any one of claims 1 to 13 for use in therapy.
35. A construct according to claim 34 for administration by particle mediated DNA delivery.
36. Use of a construct as claimed in any one of claims 1 to 13 as a vaccine.

37. Use according to claim 36 wherein the construct is administered by particle mediated DNA delivery.

Abstract

The present invention provides novel DNA constructs comprising a transcriptional regulatory sequence comprising a polynucleotide derivable from the eIF4A1 gene promoter. In preferred embodiments, the polynucleotide further comprises a polynucleotide derivable from the eIF4A gene introns, particularly intron 1. Host cells harbouring the constructs are also provided. These novel constructs have applications in gene therapy, DNA vaccines and in the commercial production of proteins.

Fig.1

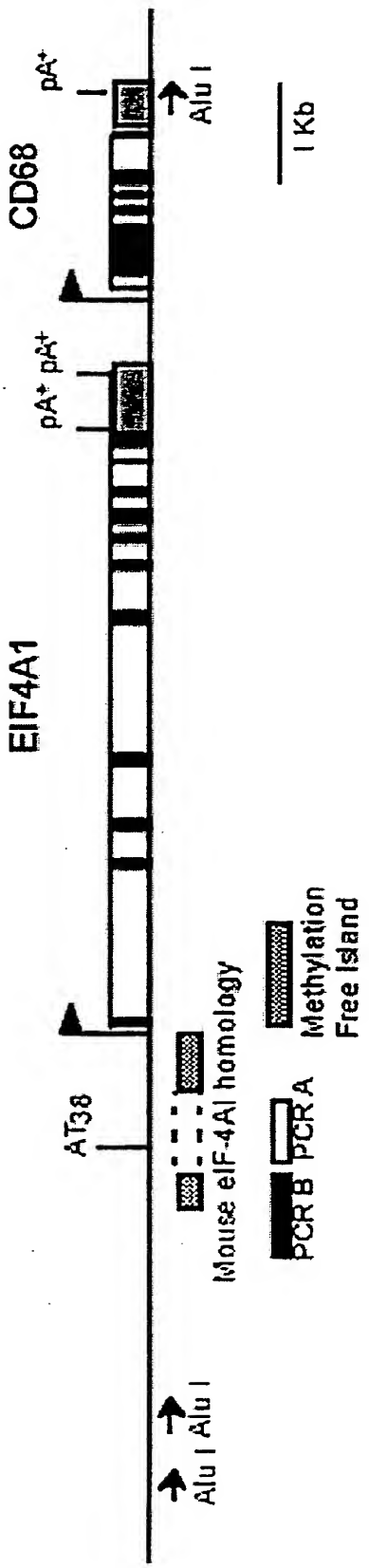

CD68
EIF4A1
pA+
pA+ pA+
Alu I
1 Kb
AT38
Mouse eIF-4AI homology
PCR B
PCR A
Methylation
Free Island
Alu I Alu I

Fig.2

-526

CTACGATATCGCTGTTGATTTCCTTCATCCCCTGGCACACGT
CCAGGCAGTGTCGAATCC

-466

ATCTCTGCTACAGGGGAAAAACAAATAACATTTGAGTCCAGT
GGAGACCGGGAGCAGAAG

-406

TAAAGGGAAGTGATAACCCCCAGAGCCCGGAAGCCTCTGG
AGGCTGAGACCTCGCCCCCC

c-ets1

Sp1

-346

TTGCGTGATAGGGCCTACGGAGCCACATGACCAAGGCACT
GTCGCCTCCGCACGTGTGAG

-286

AGTGCAGGGCCCCAAGATGGCTGCCAGGCCTCGAGGCCTG
ACTCTTCTATGTCACTTCCG

c-
ets/Elk-1

-226

TACCGGCGAGAAAGGCGGGCCCTCCAGCCAATGAGGCTGC
GGGGCGGGCCTTCACCTTGA

Sp1

-166

TAGGCACTCGAGTTATCCAATGGTGCCTGCGGGCCGGAGC
GACTAGGAACTAACGTCATG

AP-2

CRE-BP

-106

CCGAGTTGCTGAGCGCCGGCAGGCGGGGCCGGGGCGGCC

AAACCAATGCGATGGCCGGGG

AP-2 Sp1 Sp1

Sp1

-46

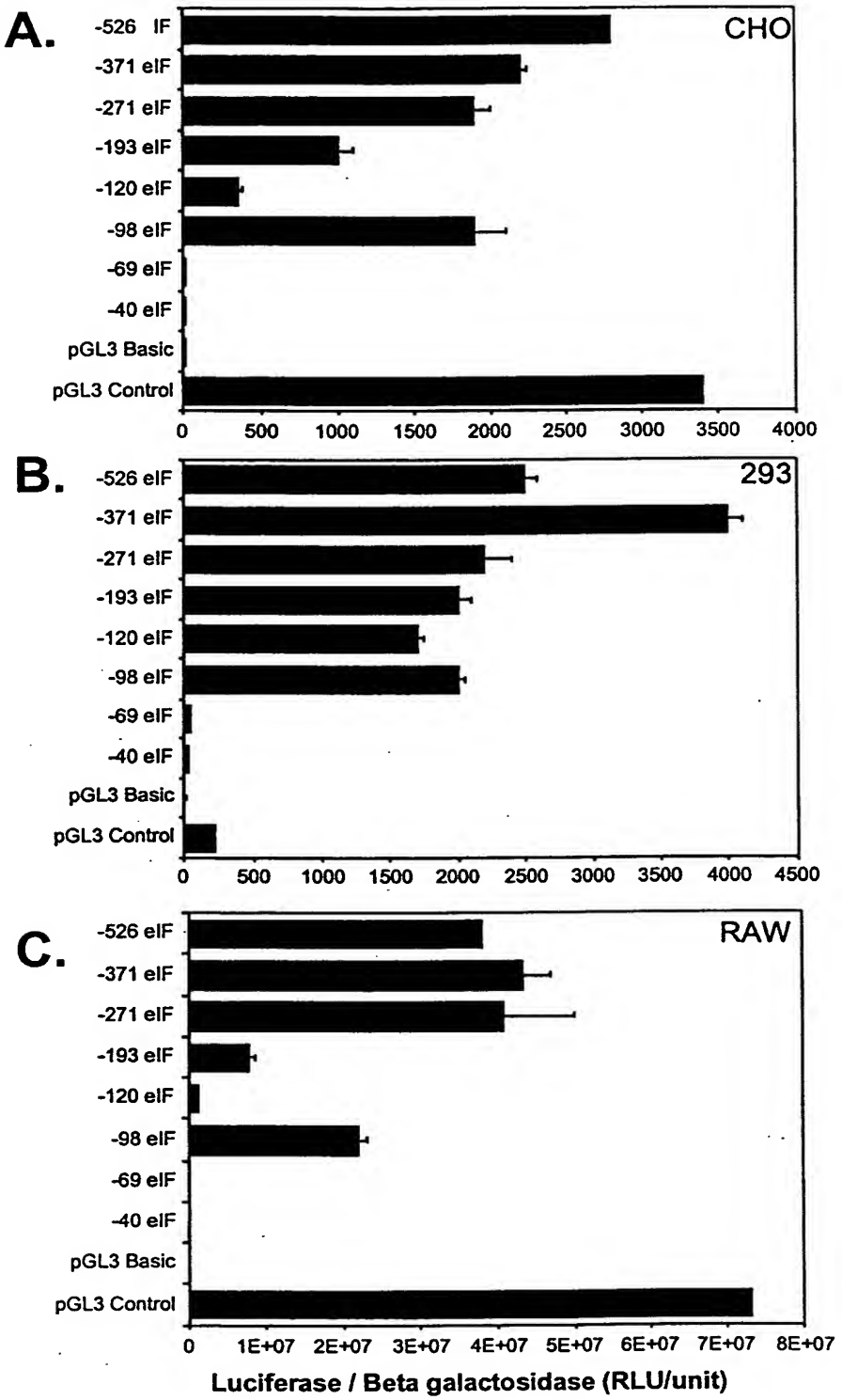
CGGAGTCGGGCGCTCTATAAGTTGTCGATGGGCGGGCACT

CCGCCCTAGTTTCTAAGGAT

+14 C ATG TCT GCG AGC CAG GAT TCC CG

met ser ala ser gln asp ser

Fig.3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CHO
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-371 eIF
-271 eIF
-193 eIF
-120 eIF
-98 eIF
-69 eIF
-40 eIF
pGL3 Basic
pGL3 Control
0 500 1000 1500 2000 2500 3000 3500 4000
B.
293
-526 eIF
-371 eIF
-271 eIF
-193 eIF
-120 eIF
-98 eIF
-69 eIF
-40 eIF
pGL3 Basic
pGL3 Control
0 500 1000 1500 2000 2500 3000 3500 4000 4500
C.
RAW
-526 eIF
-371 eIF
-271 eIF
-193 eIF
-120 eIF
-98 eIF
-69 eIF
-40 eIF
pGL3 Basic
pGL3 Control
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Luciferase / Beta galactosidase (RLU/unit)

Fig.4

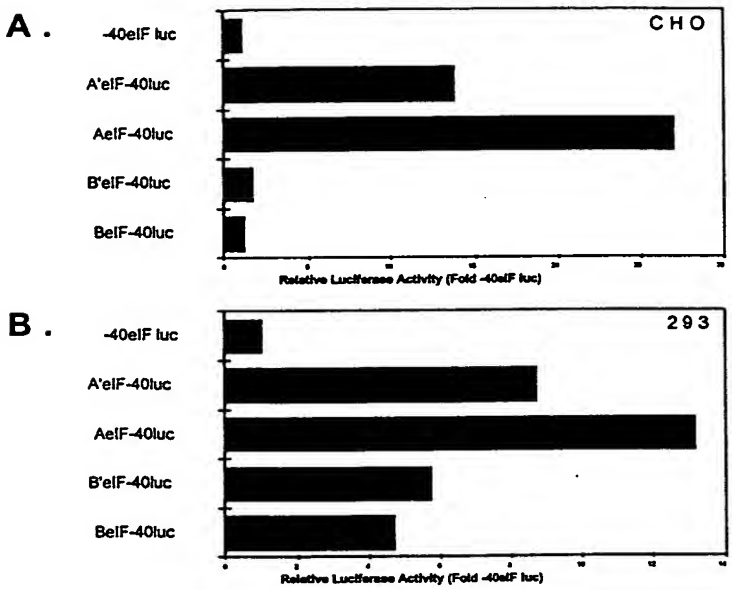
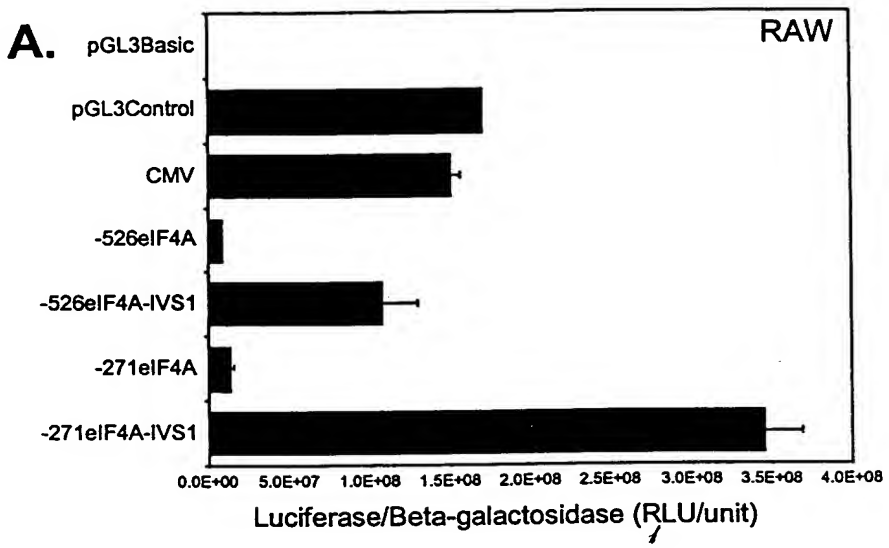

A.
-40eIF luc
A'eIF-40luc
AeIF-40luc
B'eIF-40luc
BeIF-40luc
CHO
Relative Luciferase Activity (Fold -40eIF luc)
B.
-40eIF luc
A'eIF-40luc
AeIF-40luc
B'eIF-40luc
BeIF-40luc
293
Relative Luciferase Activity (Fold -40eIF luc)

Fig.5


A.
RAW
pGL3Basic
pGL3Control
CMV
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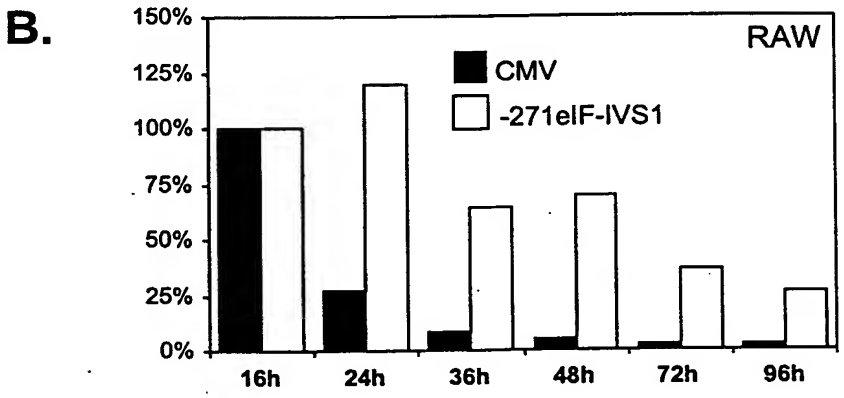
-526eIF4A-IVS1
-271eIF4A
-271eIF4A-IVS1
0.0E+00
5.0E+07
1.0E+08
1.5E+08
2.0E+08
2.5E+08
3.0E+08
3.5E+08
4.0E+08
Luciferase/Beta-galactosidase (RLU/unit)


B.
RAW
CMV
-271eIF-IVS1
150%
125%
100%
75%
50%
25%
0%
16h
24h
36h
48h
72h
96h

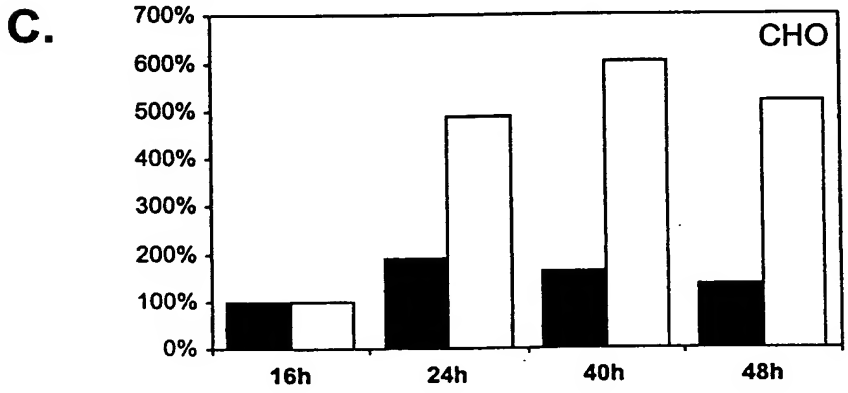
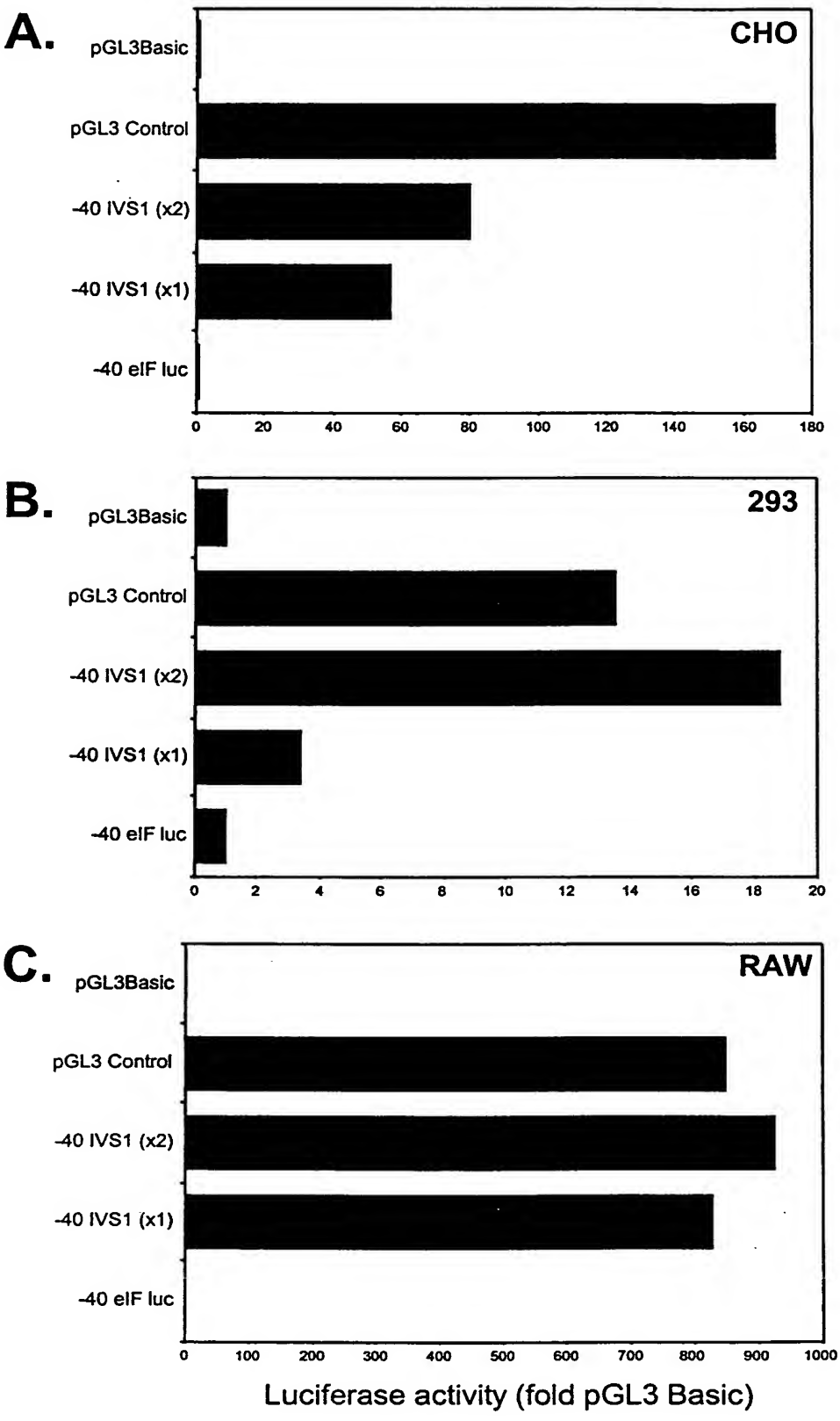

C.
CHO
700%
600%
500%
400%
300%
200%
100%
0%
16h
24h
40h
48h

Fig.6


A.
CHO
pGL3Basic
pGL3 Control
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-40 IVS1 (x1)
-40 eIF luc
0
20
40
60
80
100
120
140
160
180
B.
293
pGL3Basic
pGL3 Control
-40 IVS1 (x2)
-40 IVS1 (x1)
-40 eIF luc
0
2
4
6
8
10
12
14
16
18
20
C.
RAW
pGL3Basic
pGL3 Control
-40 IVS1 (x2)
-40 IVS1 (x1)
-40 eIF luc
0
100
200
300
400
500
600
700
800
900
1000
Luciferase activity (fold pGL3 Basic)

Fig. 7

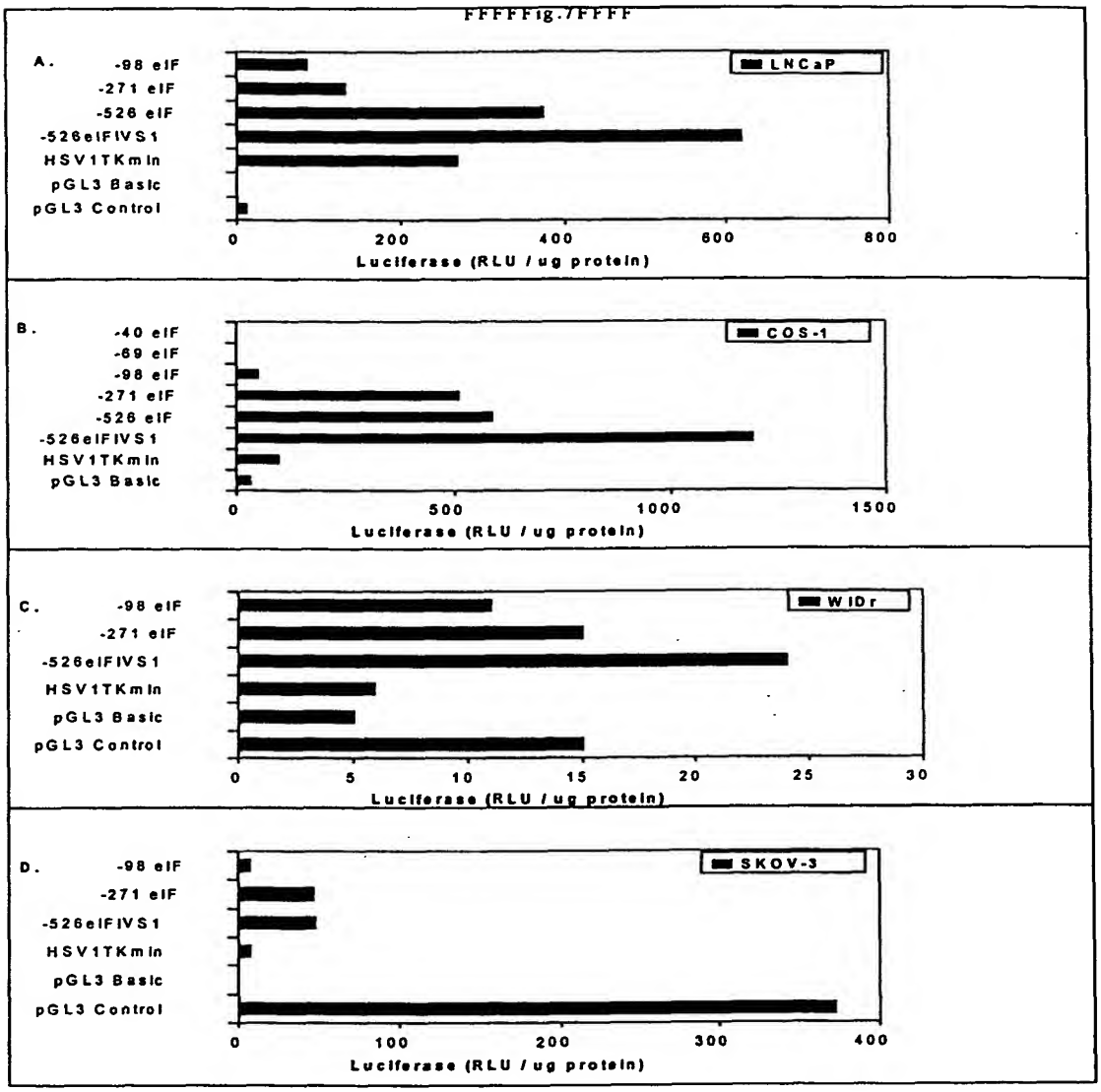
FFFFFig.7FFFF
A.
-98 eIF
-271 eIF
-526 eIF
-526eIFIVS1
HSV1TKmin
pGL3 Basic
pGL3 Control
LNCaP
0
200
400
600
800
Luciferase (RLU / ug protein)
B.
-40 eIF
-69 eIF
-98 eIF
-271 eIF
-526 eIF
-526eIFIVS1
HSV1TKmin
pGL3 Basic
COS-1
0
500
1000
1500
Luciferase (RLU / ug protein)
C.
-98 eIF
-271 eIF
-526eIFIVS1
HSV1TKmin
pGL3 Basic
pGL3 Control
WIDr
0
5
10
15
20
25
30
Luciferase (RLU / ug protein)
D.
-98 eIF
-271 eIF
-526eIFIVS1
HSV1TKmin
pGL3 Basic
pGL3 Control
SKOV-3
0
100
200
300
400
Luciferase (RLU / ug protein)

Fig.8

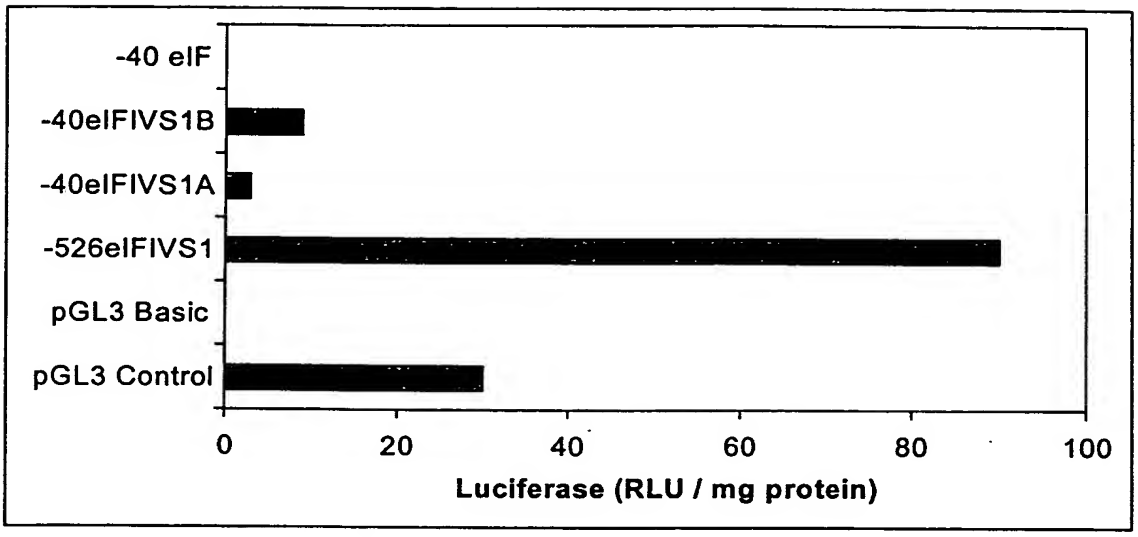
-40 eIF
-40eIFIVS1B
-40eIFIVS1A
-526eIFIVS1
pGL3 Basic
pGL3 Control
0
20
40
60
80
100
Luciferase (RLU / mg protein)

Fig.9

-5280 GCGGCCGCAT AATACGACTC ACTATAGGGA TCTAGGAAGG CCTCTCATAG

-5230 CTGAGACGTG AATGATGAGC AGCCAGCCAT GCGCAGACCT GGGAATAGCA

-5180 AGTACACAAG ACCCATAGTG AAAAACCATG GCTGAGGAAC AGAGGGCTTG

-5130 TGGGGGTGAC CTGTGTAGTT GGCGCAGAGT GAGCAAAGGG AGATGGATAC

-5080 AAAATTCGGT CAGAGAGTAG ATCATGTAAG ACATGTACGG TAGGCTGAGG

-5030 AGGGGGGATT TTATTGCGTG TATACTGAGA AGCCATTGAG TTTTAAGCAG

-4980 GCTGAGAAGT GCCTTCTGTT TTAAACTCCT GTTTCAATGA CAGATTGAAA

-4930 GGGGGGCAAG AATGGAAGCA GGAACAGAGC ACAGTAGTCC AGGTGAGAAA

-4880 CTTGAACTGG AGTGCTAAAG GAAGAGAGAG AGAGTAGTTT TATGTAGGAT

-4830 AAATTTTACG AGTAAAACCA GTAGGACTGA CAGGCTCTGT GATACTGAGA

-4780 GATACATATT TGTCTCCTGA CCAGGCTCCT GGCATTCAAC TTCTAAAATC

-4730 CTTGGAATCT CCAGTGATGT GTGTTTTTGT GTGCTGATGA GTTGATTCAT

-4680 GGCTAGCCCC TCTAGGTGGC TTCATGATTA GAGGGTTGGA ACTTTCAGCC

-4630 TCACCCCCAC CAACTTCCTG GGAGGGGAAT GGGGCCAAAG GTTAAGGCAA

-4580 TCACTGAGGA TCAGTGATTT AATCAGTCAT GCCTAGTAGT GAAGCCTCTA

-4530 AAAACCGGAA AGGGGCCGGG TTGCGCGGCG CACGCCTGTA CTCCCGGCAC

-4480 TTTGGGAGGC TGAGGCAGAT GGATCGCAAG GTCAAGAGAT TGAGACCAGC

Alu I repeat

-4430 CTGGCCGACC TGGCGAAACC CTGTCTCTAC TAAAAATATG AAAATTAGCT

-4380 GGGCGTGGTG CGTGCGCCTG TAGTCCCCGG GAGGCTGAGG CAGGAGAATC

-4330 GCTTGAACCC GGGAGGCAGA GGTTGCAGTG AGCCGAGATT GTGCCACTGC

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-4230 ACCCGAGAGG AGGAGTTTGG AGACATTCTA GATAGCTGAA GGCATGGAGG

-4180 CTGCCCACAG GATGGTCTGC CAGGCCTCTT CCCGGTACCT TTCCCTGTGC

-4130 ATCTTTTCAT CTGTACTCTT TGTACTACCC TTTGTTAATA AACTGGTAAA

-4080 TGTGTTTCCA TGAGTTCTGT GAGCTGCTCT AACAAATTAA TCAAATTCAA

-4030 GGAGGGGGTC ATGGGAACGC TGATCTAACC AGTTGGTGAG AAACACAGAT

-3980 AAAACAACCT GGGGCTTACG ACTGGCATCA GAATTGGGGG CAGCCTTGTG

-3930 AGACTGAGCC CTAAACCTGT GACACATTAT CTCCAGGTAG ATAGTGTTGG

-3880 AATTGAATTG GGGGATACCC AGCTGTGTCC ACCGCAAAAT TGCTTGCTTG

-3830 GTTGTTGGTG GAGAGAAAGC CCCACAAACA CTTCTTGGTG ACCACAGGTT

-3780 ACAGAAGTAT TTTGTGTTGT GAGAGTATAG TAGGAAAGAA GATTTGTTTT

-3730 TTTGCCGGGC GCGGTGGCTC ACGCCTGTAA TCCCAGCCCT TTGGGAGGCT

-3680 GAGGCGGGCG GATCACCAGG TCAGGAGATC AAGACCATCC TGGCTAACAC

-3630 AGTGAAACCC TGTCTCTACT AAAAATACAA AAAATTAGCC GGGCGAGGTG

Alu I repeat

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-3430 AAAAGATTTG TTTTTTCCTC TGCAGGTTGG ATGTGGGAAA TGAAGAAAAA

-3380 GAAATGGAGG ATGATGCCTA GGTTTTTGGC CTATGTAACG GGAAAAGTGG

-3330 GAGAGGAACA GGTTGGGGGA GGAAAATGAA GAGTTCTTTT CTCTTCTGGT

-3280 TTCCCTGCCC TCCCATTCAA AAGCCAGGAA ATTTCTACAG CTAGGCAGGA

-3230 TGATTGGCTC CGGCATTCCT TAATTTCAGT CCTCAAAATC AAGAGCTTAC

-3180 ACCCTCAGGG ATCTTCTTGC AGTAGAGGGA AGGGTGGTGA CGTACAGTGA

-3130 AAAACATGTT GGCCTTCTTC ATACTGAGTT TGAGTCCCAC TTCTGCCATT

-3080 TCTTTCTTTC ATGACCTTGT GCAAGTCACG ACTTTCCAAG CTGCAATTTC

-3030 CTCATCTGTT AGGTTGAATG TTGAGAACTT CCCGGTAGGA TTGTTATGAG

-2980 CATTAACTGC GTGTTTACTT TGTGCTGTGT CTTGTTCTAA GTGTATTATG

-2930 GATAGTCACT AGTTTAATCC TCATATCAAA TGGATGAGGT GTAGGTACTA

-2880 CTATTTACAC TCTCTGACAG ATAAGGAAAC TGAGGTATAG AAGGTTATTA

-2830 AGTAGGTTGC CCACTGTCAT AAGCCAGTAA ATGGAGGAGC TGTATTTGAA

-2780 TTCTGGCAGG CTCCAGAATC CTGGGCCTGG GTTCTTAGCT GCTAAGTGCT

-2730 TCTCCCTTTA AAGTGTGAAA AGCGCCTGCC CATCATGGGT TCTCAAGTGT

-2680 TCGTTCTGAT GTCTCCTCCA TTGTCTGACC TTCCTCCCTT ACCCCGAAGA

-2630 ACCGAAACAT GCAGATCCTG AGCTTGCCCA CAATCTAGGC CTTGGGTCTT

-2580 CTGTTCTTTC ACTTGGTTCC CTTACCTGTG TCTCTGTTCC TCTCTAGAAC

-2530 CTTCATGGCA AAAGGCAAGA CTTCTGTTTG TTGTACCTGA CCTGTGGCAC

-2480 TATCTCTTTA GGTGGACATC TTCAATAAGG AGCTACTGCT AATCCCCATC

-2430 CACCTGGAGG TGCATTGGTC CCTCATCTCT GTTGATGTGA GGCGACGCAC

-2380 CATCACCTAT TTTGACTCGC AGCGTACCCT AAACCGCCGC TGCCCTAAGG

-2330 TTTGAGGGGG TAGGAGAGAG ATGGGCAAAA TGTGGGGCGG TGCAGTGGCA

-2280 AGGCATTGCA GGAAGAAGGG TGGGCTTTGG GTCTTTGAGG GGCGACCTGG

-2230 GCATGGTGTC TGCCAGCACT GTACCCACCA TACTGTGTTC AATTGAGAAA

-2180 CTTAGGGCAT CACTTTCTTT TCCCCCATCC ACATAGCATA TTGCCAAGTA

-2130 TCTACAGGCA GAGGCGGTAA AGAAAGACCG ACTGGATTTC CACCAGGGCT

-2080 GGAAAGGTTA CTTCAAAATG GTGAGTTTCC TGAGGGAGGG GTATAGGGTG

-2030 TTGGTGGGGA CAGTGGTAGA AGGCAGAAAT TGAAGTCCTA CCCCTGGGAG

-1980 TCTCCATGTG AAGGGCCTGC TTTCTTTCTC TTCTCTAGAA TGTGGCCAGG

-1930 CAGAATAATG ACAGTGACTG TGGTGCTTTT GTGTTGCAGG TAAGCAGATG

-1880 ATGGGGCCAC CTCCTCTAGC TCTGAAGTCA GTTGGGTTAA AGGGTCGGGA

-1830 GGCTGTTATG CATCCCCTCA TTTGGCTCAT AGTCAGTTGT GGAGCAGGAA

-1780 GTAATCTGTT TTAGAACACC AAAACACTGG CTTCACTGGT TCTCTTCTGG

-1730 ACTTCTCCAT CCCACATTGG GACTGGGTCT CTAGGTCTTT TGGCTCTGGC

-1680 CTTCATAGAG CTCCCTGCTA ACCTCCAACT CAGTGTATTT TCTCCATCTA

-1630 AAACATTCTA TCAAGTAAGA ACACTAGCTT TAGAGTCAGG CTGTTTTTGA

-1580 ACCCCAGGCT GTGGGACCCT GGCTCCCTTT GGGGATGTTC TCTGAAGGAT

-1530 GGAGACACAT CTCATATGAA ATGTGTAGCA CAGGTCCTGA CACGGGGGGT

-1480 TTCTCATGGC TTGCTTTGTT AACACCCAGT ACTGCAAGCA TCTGGCCCTG

-1430 TCTCAGCCAT TCAGCTTCAC CCAGCAGGAC ATGCCCAAAC TTCGTCGGCA

-1380 GATCTACAAG GAGCTGTGTC ACTGCAAACT CACTGTGTGA GCCTCGTACC

-1330 CCAGACCCCA AGCCCATAAA TGGGAAGGGA GACATGGGAG TCCCTTCCCA

Homology to mouse eIF4A gene sequence

-1280 AGAAACTCCA GTTCCTTTCC TCTCTTGCCT CTTCCCACTC ACTTCCCTTT

-1230 GGTTTTTCAT ATTTAAATGT TTCAATTTCG TGTATTTTTT TTTCTTTGAG

-1180 AGAATACTTG TNTATTTCTG ATGTGCAGGG GATGGCTACA GAAAAGCCCC

-1130 TTTCTTCCTC TGTTTGCAGG *GGAGTGTGGC CCTGTGGCCC TGGGTGGAGC*

-1080 *AGTCATCCTC CCCCTTCCCC GTGCAGGGAG CAGGAAWTCA GNGATGGGGG*

-1030 *GNGGGGGGCG GACAATAGGA TNACAGCCCG CCAGATATNC ATATATATAT*

-980 *ATATATATAT ATATATATAT ATATATATAT ATATATATAT ATATATATAT*

-930 *ATATATATAT ATATATAAAA ATGCCACGGT CCTGCTCTGG TCAATAAAGG*

-880 *ATCCTTTGTT GATACGTAAG TGGTGGTCTT CCTTAAGGGG CTTCAAATTA*

-830 *GTGGATATGC TTAGCTCAGA CCTTCCAGCC AGTNTCTTGA GACTAAAGGG*

-780 *TTCAGCTTTC CATCCCTGGC TCAGGCACTG CCAACACCTT GTCTTCACCC*

-730 *AAACAAATCC CCCAGATGGG AGCAGAGAGC AGGAAGGAGG GAAAGTAGAT*

-680 *AAGCCTCAAG AATAAGGGCA TCCGAGAGGG AAGCGTGGGG AACTGGACAC*

-630 *AAGGGACTGG GGAGGGGACC AACCAGGATT CATGATAGTA CCCCAAAGCC*

-580 *CTTTACAGTT TTYTTCCATC CCTCCACCAT CCAGCCAGGG GAATCCTCCC*

-530 *ATCCCTACGA TATCGCTGTT* GATTTCCTTC ATCCCCTGGC ACACGTCCAG

-480 GCAGTGTCGA ATCCATCTCT GCTACAGGGG AAAAACAAAT AACATTTGAG

-430 TCCAGTGGAG ACCGGGAGCA GAAGTAAAGG GAAGTGATAA CCCCCAGAGC

-380 CCGGAAGCCT CTGGAGGCTG AGACCTCGCC CCCCTTGCGT GATAGGGCCT

-330 ACGGAGCCAC ATGACCAAGG CACTGTCGCC TCCGCACGTG TGAGAGTGCA

-280 GGGCCCCAAG ATGGCTGCCA GGCCTCGAGG CCTGACTCTT CTATGTCACT

-230 TCCGTACCGG CGAGAAAGGC GGGCCCTCCA GCCAATGAGG CTGCGGGGCG

Homology to mouse eIF4A gene sequence

-180 GGCCTTCACC TTGATAGGCA CTCGAGTTAT CCAATGGTGC CTGCGGGCCG

-130 GAGCGACTAG GAACTAACGT CATGCCGAGT TGCTGAGCGC CGGCAGGCGG

-80 GGCCGGGGCG GCCAAACCAA TGCGATGGCC GGGGCGGAGT CGGGCGCTCT

-30 ATAAGTTGTC GATGGGCGGG CACTCCGCCC TAGTTTCTAA GGATCATGTC

+20 TGCGAGCCAG GATTCCCG

Fig. 10

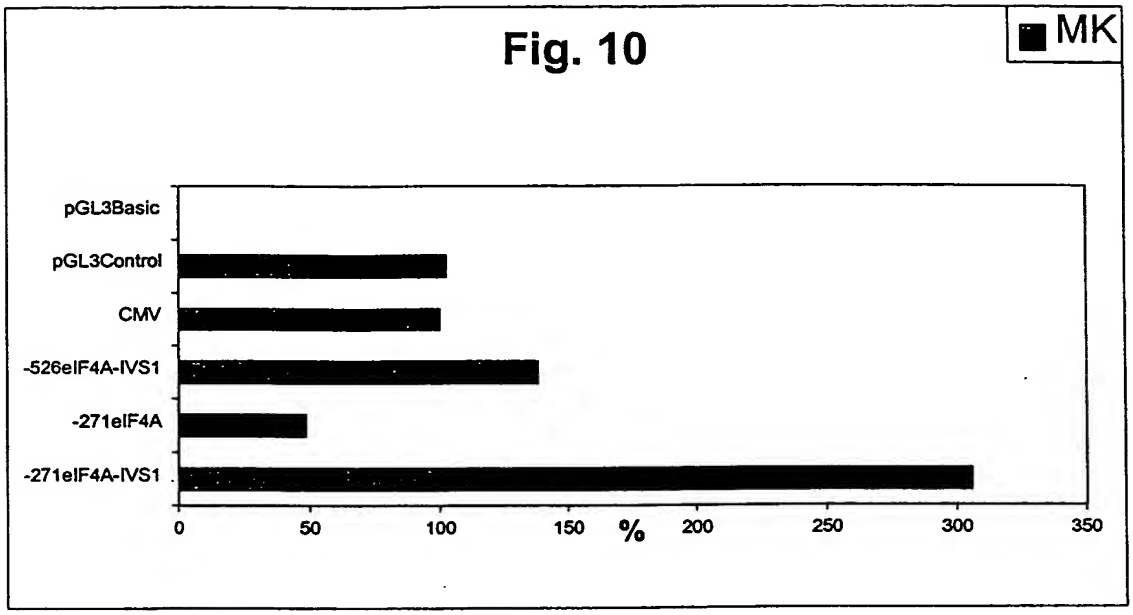


Fig. 10
MK
pGL3Basic
pGL3Control
CMV
-526eIF4A-IVS1
-271eIF4A
-271eIF4A-IVS1
0
50
100
150
%
200
250
300
350

Fig. 11

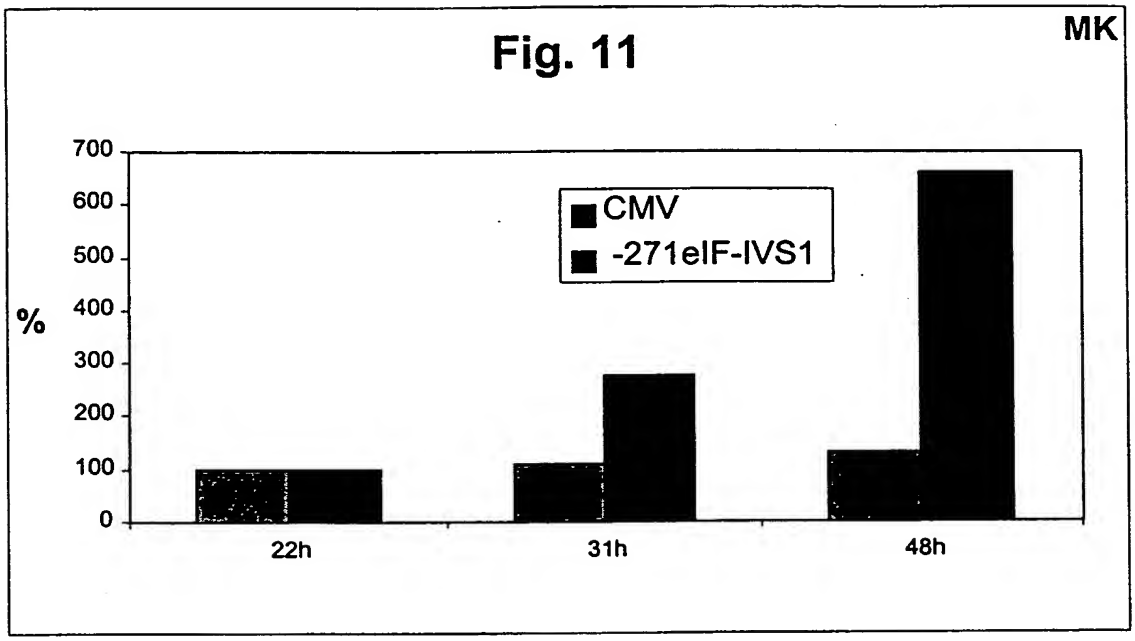


Fig. 11
MK
700
600
500
400
300
200
100
0
%
CMV
-271eIF-IVS1
22h
31h
48h

Fig. 12

Figure 12

99.31.2plot2

11

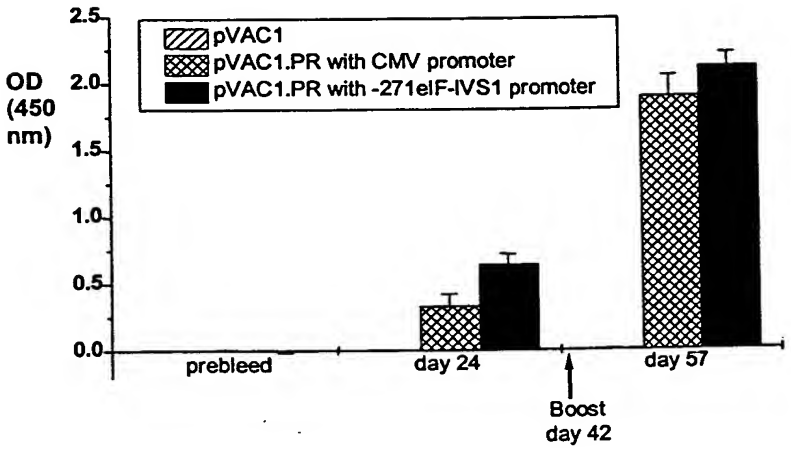
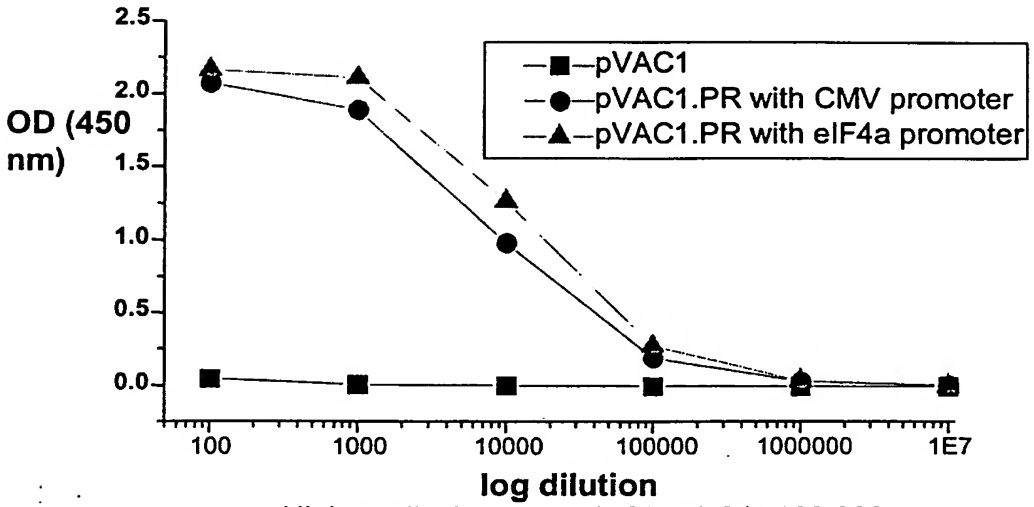


Fig. 13

Figure 13



Highest dilution to reach OD >0.2 is 100,000
for both CMV and -271eIF-IVS1 promoters

PCT/GB 00/02569

23/74 filed 5-7-2000

Glaxo Wellcome PLC